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**A THESIS
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY**

**Transcriptomic analysis of useful genes applicable
for RNAi-based control of the small brown
planthopper and rice stripe virus**

전사체 분석을 통한 유용 유전자의 RNAi 를 활용한
애멸구 및 벼줄무늬잎마름바이러스의 방제

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February, 2018

**Transcriptomic analysis of useful genes applicable for RNAi-
based control of the small brown planthopper and rice stripe virus**

**UNDER THE DIRECTION OF ADVISER YEON HO JE
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL OF
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ABSTRACT

The small brown planthopper (SBPH), *Laodelphax striatellus* (Fallén), is an economically important phytophagous species in the family of Delphacidae of Hemiptera. It has a wide distribution range from south-east Asia to Siberia and Europe, and attacks several important agricultural crops including rice, corn, wheat, oat and barley. SBPH is one of the most serious pest insects of rice plants because it transmits the rice stripe virus (RSV) in a circulative-propagative manner which typical symptoms include pale and

discontinuous yellow stripes, blotches and dead tissue streaks on the leaves. Chemical insecticides have been used to this economic pest for several decades.

Buprofezin is an insect growth regulator (IGR) pesticide which is active against larval stages to cause cuticular lesions that result in the disruption of chitin synthesis. Because this insecticide has generally been considered to have a good efficacy against the target pests while being harmless to beneficial insects, it has been used widely in integrated pest management programs especially used in the control of homopteran pests. As buprofezin used to control *L. striatellus* for more than a decade, the buprofezin resistant occurrence of *L. striatellus* was reported recently.

To survey the responses of SBPH to buprofezin, transcriptome analysis of buprofezin treated SBPH had been investigated. 200ppm buprofezin was exposed to 4th instar SBPH by the dipping method, and extracted total RNA for RNA-seq by Illumina platform. Total of 2 x 26,848,684 and 2 x 27,310,742 of 101 base paired-end raw reads were obtained from buprofezin treated SBPH and control samples, respectively. The cDNA library containing 23,817 contigs was constructed by Trinity *de novo* assembler and TransDecoder ORF finder. The quality filtered raw reads from experimental and control SBPH were subjected to the library with Bowtie2 and eXpress computer programs to obtain the differential gene expression profile. Following analysis revealed that 170 and 144 contigs were down- and up-regulated over five-folds in the buprofezin treated samples, respectively. GO (gene ontology) enrichment analysis were subjected to study the changes of gene expression profile. Total of 9 GO terms showed differential expression under buprofezin treatment. These transcriptome and gene expression

profiling data will provide important information for future studies of molecular biology and physiology of *L. striatellus*.

To develop an alternative pest control strategy, six buprofezin-specific genes selected by differential expressed genes from RNA-seq of buprofezin-treated *L. striatellus* were synthesized to dsRNAs, and applied to *L. striatellus* to assess the insecticidal efficacy. Two and three of those dsRNAs showed moderated and substantial insecticidal activity up to 60% of mortality in 7 days post treatment, respectively. These results demonstrated the potential of gene screening strategy for the development of RNAi-based pest management program.

RNA interference (RNAi) was not only suggested as a promising strategy for controlling insect pests, but also affects the transmission of plant pathogens of insects. To disturb transmission of the RSV in SBPH by using RNAi pathway, we chose nine genes highly expressed in RSV-viruliferous SBPH by transcriptome sequencing. These SBPH-derived dsRNAs were applied to the insects indirectly through xylem of rice leaves by irrigation. qPCR result demonstrated that three out of eight SBPH-derived dsRNAs successfully reduced the replication of RSV in viruliferous SBPH in dose-dependent manner, suggesting that these three dsRNAs could suppress replication of RSV and provide a new tool for RSV control strategy.

In summary, an effective high-throughput NGS method largely broadens the target selection of specific RNAi genes from the non-model pest *L. striatellus*. It may lead to new strategy in designing the RNAi-based technology against SBPH damage and rice stripe virus transmission by *L. striatellus*.

Key words: Rice stripe virus, *Laodelphax striatellus*, buprofezin, RNA-seq, RNA interference, double-stranded RNA

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LITERATURE REVIEW

1. Basic biology of *Laodelphax striatellus*

Laodelphax striatellus (Fallén) (small brown planthopper, SBPH), *Nilaparvata lugens* (Stål) (brown planthopper, BPH), and *Sogatella furcifera* (Horvath) (whitebacked planthopper, WBPH) have become major pests successively in the rice-growing areas of Asia and extensive studies have been carried out to develop control programs since the 1960s.

The small brown planthopper, *Laodelphax striatellus* (Fallén) (Hemiptera:Delphacidae) is one of the most important pests that causes virus disease to rice paddy fields by transmitting the plant-pathogenic virus. It is known to transmit viruses to over 80 host plants. For this reason, controlling the rice stripe virus is almost impossible since the virus can survive at many different hosts even after all the rice get harvested. SBPH is mainly found in the Northeast Asian countries such as China, Japan, Taiwan and Korea and is considered as a most economic pest in the rice field.

The SBPH is dimorphic, with fully winged 'macropterous' and truncate-winged 'brachypterous' forms. The macropterous forms can migrate when the insect faces a lack of food or inconvenient circumstances. Once the SBPH settles down on the rice, they produce the next generation in which almost all female insects develop as brachypters and males as macropters. Adults usually mate on the day of emergence, and the females

usually lay eggs from the day following mating. Brachypterous females lay 300 to 350 eggs, while macropterous females lay fewer eggs. Eggs hatch in about six to nine days. The newly hatched nymphs are cottony white, and turn purple brown within an hour; they feed on the plant sap, and undergo five instars to become adults.

The SBPH overwinter in fourth instar nymph in the soil, root of wheat, stubble, fallen leaves, weeds and other places. The length of the life cycle is variable; only one generation per year in northern countries while up to 5 generations have been reported in southern countries. They can overwinter in Korea and often come flying from abroad. The eggs hatch in about 7-10 days and then live as nymphs for eighteen to twenty days. After the five instars they become adults and can live for 20 to 25 days. The SBPH normally produces five generations per year. The first generation starts from the middle of March which has overwintered. The second generation is the most important for pest control since it moves out from wheat field to the early rice paddy field which is very susceptible to pathogens. This generation is responsible the outbreaks of SBPH. The forth instar nymph of fifth generation begins to overwinter in November.

The body size of adult females and males are approximately 3.6-3.8 mm and 3.4-3.6 mm in length, respectively. The body of adult SBPH is black to dark-brown, and the coloration varies with season. The mesonotum is black to dark-brown, as well as the pterostigma on the fore wings, and the areas between the carinae of the frons are deep black. The head is lemon yellow, and most of the compound eyes are black but often red while a stemma is dark-reddish.

2. General characteristics of Rice Stripe Virus

Rice stripe virus (RSV) causes a severe disease of rice in Asian countries and mainly occurs from mid-May to early June. RSV is a plant pathogenic virus of the genus *Tenuivirus*. Other members of this genera are Maize stripe virus, Rice hoja blanca virus, and Iranian wheat stripe virus. The *Tenuivirus* causes diseases in their host plants with typical symptoms of chlorotic stripes on the affected leaves, and the infected rice plants produce no grain when they are inoculated earlier than the leaf stage.

The genome of RSV comprises four species of segmented single-stranded RNA genome of RNA1, 2, 3 and 4 according to the size of each RNA molecule (Hibino 1985, Ishikawa 1989). The largest segment RNA1 encodes a 336.8 kDa protein which is thought to be a RNA dependent RNA polymerase (Toriyama 1994). RNA2 encodes 94 kDa glycoprotein and NS2 (Takahashi 1993), RNA3 encodes NS2 and CP (Zhu 1991), and RNA4 encodes SP and NS4 (Zhu 1992). RNA1 is negative-sense single strand while RNA2, 3, and 4 are ambisense in their coding strategy (Takahashi 1993).

Hemipteran insect have predominant features that allow the efficient virus transmission. The distinct feature of these insects is that they have piercing-sucking mouthparts which include a needle-like stylet bundle compounding of two maxillary and two mandibular stylets. The two maxillary stylets are interlocked and form two canals. While the wider food canal takes up plant sap into the cibarium, the esophagus, and the rest of the alimentary canal, the narrower salivary canal delivers saliva with the virus into the feeding puncture in the plant tissues.

There are four described mechanisms of insect transmission of plant-pathogenic viruses; nonpersistent, persistent-circulative, persistent-propagative, and semipersistent. While in nonpersistent transmission, insects can inoculate the virus into plants for only a few minutes after the acquisition and the insect loses the virus within a few minutes and upon molting. In persistent transmission, vector insects can inoculate the acquired virus for much longer periods from days to week, transmitting the virus after molting and often for their entire lifespan. The SBPH transmits the RSV in a persistent-propagative manner which means the viruses are often transmitted to the vector's progeny through the infection of the embryos or germ cells in the female insects (Sylvester 1980). Also, evidences have revealed that amorphous or filamentous inclusions of RSV genes exist in most of the insect tissues (Zhang 2010) by detection of RSV gene products by light microscopy. Moreover, it has been confirmed that RSV particles which exist in follicular cells of the ovarioles can be transmitted from female adults to their progeny via eggs (Liang 2005).

3. RNA-Sequencing

After completed the human genome project in 2003, sequencing technology has been rapidly changed. The automated Sanger method is considered as a 'first-generation' technology, and newer methods are referred to as Next generation sequencing (NGS). From 2007, term of NGS has been used. NGS technologies revolutionized genomics and their effects are becoming increasingly widespread. The use of NGS over the past five

years has revolutionized the discovery of microorganism (Liu 2011) and the technology allows saving the money and time. NGS not only changed the sequencing technology conveniently but also made a new platform of genome research.

Recently, the development of next generation sequencing (NGS) technologies provides a powerful alternative strategy for transcriptome analysis. The NGS technologies such as 454 GS FLX (Roche), Genome Analyzer (Illumina) and AB SOLiD (ABI) were developed to derive not only an accurate and quantitative measure of individual gene expression, but also to discover novel transcribed regions and the alternative splice events. On all three of the platforms, genomic DNA or cDNA fragments are sequenced through massively parallel DNA-sequencing approach, producing large numbers of relatively short reads of tags (Wang 2009). Read lengths range from 30-100bp for Illumina GA and ABI SOLiD to 200-500 bp for GS FLX. Although these systems were significantly different in the approached used to produce massive amounts of sequences, these platforms were similar to rely on the work flow for the production and analysis of sequencing libraries.

In sequencing reaction, NGS technologies exploit light emitted when the correct base matches the template being sequenced at every sequencing cycle. In each cycle, the flow cell is imaged in the number of non-overlapping regions. NGS technologies have an impressive application in a wide variety of fields such as solving practical challenges in medicine, engineering, agriculture and ecology (Ansorge 2009).

4. RNA interference

Nearly 10 years ago, Fire described a process in which the application of exogenous dsRNA silenced the homolog endogenous mRNA in the worm *Caenorhabditis elegans* and called it RNA interference (RNAi) (Fire 1998). Although new for animals, the technique was already described as ‘post-transcriptional gene silencing’ in plants and as ‘quelling’ in fungi. Moreover, those three techniques appeared to be remarkably well conserved in several eukaryotes (Fire 2007). RNAi soon proved to be very promising in several research fields: in genomics for gene function determination and gene knockdown in eukaryotes and in medicine to control cancers and viral disease.

The mechanism of cell-autonomous RNAi is best known: dsRNA is cleaved by an RNase III, often called Dicer, into 21–25 bp short interfering RNA duplexes (siRNA). These siRNAs are incorporated in the RNA induced silencing complex (RISC); after discarding the passenger strand, the RISC will bind to a homolog mRNA, cutting it and thereby hindering translation. A number of factors determine whether a strong silencing effect can be achieved. First, dsRNA has to be taken up into the target cell in order to elicit a response. Second, this dsRNA must be processed by the RNAi machinery within the cell, eventually creating secondary 20–25 bp siRNA molecules that can act as triggers for sequence specific enzymatic degradation of endogenous mRNA.

The dsRNA is delivered either by injection or by feeding insects. It has been reported that in several insect orders the gene can be knocked down by dsRNA injection (Tomoyasu and Denell 2004, Dong and Friedrich 2005, Mart ín 2006, Mutti 2006), but injection is not applicable to control pest insects in the field. Since then, it was reported in

a few insect species that dsRNA can be integrated in artificial diets and results in target gene knockdown (Araujo 2006, Turner 2006). In addition, two research papers indicate that transgenic crops can produce dsRNAs to protect the plant against insect feeding damage (Mao 2007). However, this technique is relatively complex and premature in terms of practical application in pest insect control. Thus, a more convenient way should be explored before dsRNA can be used as insecticides.

Regarding the optimal target gene selection, taking advantage of the known genes and cDNA library screening were the main ways up to now (Araujo 2006, Turner 2006, Baum 2007, Whyard 2009). Herein, the selection of target genes from the considered pest insects is still a major challenge. A high throughput screening system of RNAi target genes will be more appreciated when RNAi-based pest insect control can be taken in consideration. Recent results suggest that the next-generation sequencing (NGS) technologies allow directly sequencing the cDNA generated from RNA-seq (Haas and Zody 2010). This new method for analyzing RNA-seq data enables the de novo reconstruction of the transcriptome for a non-model organism (Kahvejian 2008) and can be used in RNAi target genes screening (Wang 2011).

CHAPTER 1. The transcriptomic responses of small brown planthopper, *Laodelphax striatellus* upon buprofezin treatment

ABSTRACT

The small brown planthopper (SBPH), *Laodelphax striatellus* (Fallén), is one of the most serious pest insects of rice plants because it transmits the rice stripe virus (RSV) which often causes significant reduction of yield in the rice field. buprofezin is an insect growth regulator (IGR) pesticide which is active against larval stages to cause cuticular lesions that result in the disruption of chitin synthesis. Because this insecticide has generally been considered to have a good efficacy against the target pests while being harmless to beneficial insects, it has been used widely in integrated pest management programs.

To survey the responses of SBPH to buprofezin, 200ppm buprofezin was exposed to 4th instar SBPH by the dipping method, and extracted total RNA for RNA-seq by Illumina platform. Total of 2 x 26,848,684 and 2 x 27,310,742 of 101 base paired-end raw reads were obtained from buprofezin treated SBPH and control samples, respectively. The cDNA

library containing 23,817 contigs was constructed by Trinity *de novo* assembler and TransDecoder ORF finder. The quality filtered raw reads from experimental and control SBPH were subjected to the library with Bowtie2 and eXpress computer programs to obtain the differential gene expression profile. Following analysis revealed that 175 and 144 contigs were down- and up-regulated over five-folds in the buprofezin treated samples, respectively. GO (gene ontology) enrichment analysis were subjected to study the changes of gene expression profile. Total of 9 GO terms showed differential expression under buprofezin treatment. These transcriptome and gene expression profiling dates will provide important information for future studies of molecular biology and physiology of *L. striatellus*.

Key words: RNA-seq, *Laodelphax striatellus*, buprofezin

1. INTRODUCTION

The small brown planthopper (SBPH), *Laodelphax striatellus* (Fallén), is an economically important phytophagous species in the family of Delphacidae of Hemiptera. It has a wide distribution range from south-east Asia to Siberia and Europe, and attacks several important agricultural crops including rice, corn, wheat, oat and barley. The damage is inflicted not only by direct feeding but also by transmitting several plant viruses such as rice stripe virus which causes chlorotic stripes or necrotic streaks on leaves, and premature wilting (Toriyama 1986, Hibino 1996, Falk and Tsai 1998). Although in Korea SBPH overwinters as the native species, recent studies revealed that SBPH is also a migratory pest. SBPH can migrate from one place to another place within China, as well as from China to Japan and Korea (Otuka 2010, Kim 2011).

Insect growth regulators (IGRs) are specific to target insects and display relatively low toxicities towards non-target species compared to other chemical insecticides. Commercially available IGRs have been divided into three main types depending on their mode of action (Pener and Dhadialla 2012). The first group is known as the juvenile hormone agonists (JHAs) which mimic juvenile hormone (JH) activity and fatally disrupt the endocrine system (Slama 1971). The second group consists of the ecdysone agonists and antagonists which interfere the action of molting hormone and hinder normal

development. The third group consists chitin synthesis inhibitors which are the common IGR insecticides against planthoppers (De Cock and Degheele 1998). In third group, buprofezin is especially used in the control of homopteran pests, such as *L. striatellus*. Its mode of action is not fully understood, although the primary effect is active against larval developmental stages, causing cuticular lesions that result from the disruption of chitin synthesis (Uchida 1985). Against *N. lugens*, efficient nymphicidal activity of buprofezin has been found even at a concentration of less than 1 ppm (ASAI 1983). Because this insecticide has generally been considered to have a good efficacy against the target pests while being harmless to beneficial insects, it has been used widely in integrated pest management (IPM) programs (Nagata 1986).

In recent years, the next-generation high-throughput RNA-sequencing techniques have been developed dramatically and improved the efficiency of discovering genes (Ansorge 2009). The Illumina sequencing makes possible the mapping of short reads to individual gene sequences, and thus the quantification of overall gene expression levels is possible by counting the number of tagged short reads. Therefore, the Illumina sequencing technology expanded its applications to genome and transcriptome sequencing, gene identification, annotation, and tagging of the mapped genes for quantitative analyses especially for

non-model species whose genome sequence is not available (Velculescu 1995, Mardis 2008, Wang 2009).

This new method for analyzing RNA-seq data has been used for *L. striatellus* for over 10 years. Transcriptome analysis between viruliferous and naive *L. striatellus* was reported (Zhang 2010, Lee 2013). Differentially responsive genes of the *L. striatellus* to low and high temperatures were analyzed using comparative genomics (Huang 2017). Organ-specific transcriptomes of the alimentary canal and salivary gland were analyzed in viruliferous and naive *L. striatellus* (Zhao 2016). The transcriptomic responses of *L. striatellus* upon juvenile hormone agonist and antagonist treatment have been reported (Fang 2017). Also, microRNA profiles of *L. striatellus* using the small RNA libraries derived from virus free and rice black-streaked dwarf virus infected insects were examined (Li 2015).

Buprofezin-resistant strain of *L. striatellus* has been sequenced (Zhang 2012), however, the differentially expressed genes of buprofezin-treated *L. striatellus* have not been reported. In this study, to investigate the impact of buprofezin in molecular level, the Illumina sequencing technology was employed to sequence the transcriptome of *L. striatellus* under buprofezin treatment, and construct a reference *in silico* cDNA sequences by *de novo* assembly (Grabherr 2011). For differential gene expression study, the *in silico*

cDNA library was constructed by Trinity *de novo* assembler. The expression levels of each gene were quantified by Bowtie2 and eXpress programs, and then subjected to gene ontology (GO) enrichment analysis to study the changes of gene expression profile.

2. MATERIALS AND METHODS

2.1 Insect rearing and buprofezin treatment

The *L. striatellus* was collected from the healthy rice (*Oryza sativa*) field and reared in the laboratory on 2–3 cm tall rice seedlings in glass vessels under a light:dark cycle of 16:8 h at 26 °C, and transferred to fresh seedlings every 10–14 days to assure sufficient nutrition. The chemical buprofezin was purchased from SigmaAldrich (N98%, purity, St. Louis, MO, USA). For the insecticide treatment, dipping solutions of 200 ppm buprofezin was prepared in DDW with 0.05% polyvinyl alcohol; and fourth instar nymphs of *L. striatellus* were dipped for 30 seconds. After dipping, water was removed with paper towel and treated nymphs were cultured in plastic tubes containing four rice stems with moistened cottons to wrap the rice roots, and the test tubes were maintained at the previously described rearing condition. The control sample was treated with DDW containing 0.05% polyvinyl alcohol only.

2.2 RNA extraction

To conduct the transcriptome sequencing of *L. striatellus* upon buprofezin treatment, total RNA samples were isolated at 6 h post treatment by using Qiazol lysis reagent

(Qiagen, Germany) according to the manufacturer's protocol. Twenty individuals of buprofezin treated 4th instar nymphs were pooled and homogenized in 1 ml of Qiazol reagent, and added 0.2 ml of chloroform. After vortexing, the sample tube was centrifuged at 12,000 g, the supernatant was transferred to a new tube, and added 0.5 ml of isopropanol for RNA precipitation. The RNA was washed with 75% ethanol and resolved in nuclease-free water, and stored -80°C for sequencing.

2.3 cDNA synthesis, sequencing and data analysis of RNA-Seq

The mRNA in the total RNA was converted into a library which was suitable for cluster generation by TruSeq RNA sample preparation kit. The first step was purifying the mRNA molecules by using poly-T attached magnetic beads followed by fragmentation. The cleaved fragments were reverse transcribed with random primers, subjected to second strand cDNA synthesis using DNA polymerase I and RNase H. These cDNA fragments were subjected to the end repair process, the addition of a single dATP, and ligation of the sequencing adapters. The products were then purified and enriched with PCR to create the final cDNA library. Finally the cDNA library of 250–300 bp insert was subjected to Illumina HiSeq 2000 sequencing platform for RNA-Seq to obtain 101 bases of paired-end sequences.

Because the genome or reference gene sequences of *L. striatellus* are not available at this moment, *de novo* assembly of the Illumina short read sequences was performed to construct the reference cDNA sequences based on the previously reported method (Fang 2017). Series of computational processes were performed on a workstation computer running 64-bit Linux OS with Intel i7 CPU and 128GB of main memory. The raw reads from RNA-Seq of each sample were pooled and filtered by using NGS QC toolkit (Patel and Jain 2012) to remove low quality (Q-score>30) reads. The filtered clean reads were subjected to Trinity *de novo* assembler v2.4 program (Grabherr 2011) to generate contigs longer than 200 bp. The Trimmomatic (Bolger 2014) command line option of the Trinity program was also applied to remove adapter and artifact sequences from the quality-filtered short reads.

The standalone version of TransDecoder program which was previously introduced with Trinity program (Haas and Papanicolaou 2016) was used to predict protein-coding sequences. Subsequently, the CD-HIT-EST (Li 2015) program was used to cluster homologous sequences with default parameters to merge highly homologous contigs (identity>95%) to construct final version of *in silico* cDNA library.

The short reads of each sample were then mapped to the cDNA library by using Bowtie2 program (Langmead and Salzberg 2012) followed by eXpress program (Roberts and

Pachter 2013) to compare the differential expression of each gene between control and buprofezin treated samples. And the cDNA library was also searched with the BLAST2GO program for BLASTX, Gene ontology and KEGG pathway analysis (Conesa 2005).

2.4 Validation of sequencing result by qPCR

To validate the RNA-Seq data with Hiseq 2000 system, the gene expression levels of each transcriptome measured by eXpress software were compared with those of qPCR. One μ g of total RNA was reverse transcribed to cDNA with the QuantiTeck[®] Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. qPCR was conducted by using QuantiTect reverse transcription kit (Qiagen, Germany), EvaGreen qPCR Mastermix (Applied Biological Materials Inc., Canada), and CFX96[™]Real-Time system (Bio-Rad, USA) according to the manufacturers' instructions. The PCR amplification profile was 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 58 °C for 15 s, and 72 °C for 30 s. The ADP ribosylation factor (ARF) was used as a reference gene for the calculation of fold change. The relative transcription levels were calculated by using the $2^{-\Delta Ct}$ method (Pfaffl 2001). The qPCR primer sequences of arbitrarily selected genes which were used for validation of NGS results are described in Table 1.

Table 1. Oligonucleotide sequences used for quantitative real-time PCR.

Gene name	Target-ID	Forward primer	Reverse Primer
39860	comp39860_c0_seq2	CTTACGACCAGGGCTACACA	ACTCCAATCCGGACTACGAC
55234	comp55234_c0_seq2	GCTTACACATTCCTTCAGCCT	TGAAATGGAAGGTTGGTGTG
56615	comp56615_c0_seq1	AGGTGGTGTGGGTAGGAAAC	TTCAGTTTGGGACTGTCAGC
57447	comp57447_c0_seq6	TTCCCATTTGGACCAGCTT	ACTTCAGTTGTCCACAAGTATCG
57884	comp57884_c0_seq7	AGTACCAGGAGACAGACGCA	TTCCCTTGCGAATAACCTC
59719	comp59719_c0_seq1	AGAGTCGAACATCTGATGATACCATAC	AGAGCTCAGAAATATTTGAGAGTAG
ARF	ADP-ribosylation	TTGGACAGTATCAAGACCCATC	GCAGCAATGTCATCAATAAGC

3. RESULTS

3.1 Illumina sequencing

The buprofezin treated *L. striatellus* nymphs showed approximately 80% of mortality at 48 h post treatment. For total RNA extraction, the nymphs were collected at 48 h post treatment when most of the nymphs were viable. The total RNA from control and buprofezin treated nymphs were subjected to Illumina HiSeq 2000 sequencer to obtain 27,301,742 and 26,848,684 bp of raw reads, respectively. By using NGS QC Tool kit, low quality sequence reads were filtered, and the percentage of the clean reads were 98.74% and 95.44%, respectively. The detailed transcriptome sequencing results of each sample was summarized in Table 2. The raw Illumina sequencing results were submitted to NABIC (National Agricultural Biotechnology Information Center, Rural Development Administration, Korea) NGS SRA database and the accession numbers for the transcriptome sequences of buprofezin treated and control sample are NN-3355 and NN-3350, respectively.

Table 2. Summary of the Illumina sequencing.

	Mock-treated	Buprofezin-treated
	<i>L.striatellus</i>	<i>L.striatellus</i>
Total paired-end sequence reads	27,301,742	26,848,684
Total paired-end clean reads	26,138,125	25,624,072
Total nucleotides of clean reads(bp)	2606,153,240	2588,031,272
Percentage of clean reads	95.74%	95.44%
GC% of clean reads	33%	38%

3.2 Characterization of the de novo assembled in silico cDNA library of *L. striatellus*

Total of 194,199 contigs were initially obtained by Trinity program from the 54,150,426 of pooled, paired-end clean reads of all the samples, and the final version of *in silico* cDNA library which contains 23,817 contigs (average length = 1,063 bp, N50 length = 1,461 bp) was obtained by using TransDecoder and CD-HIT-EST program. The length distributions of the *L. striatellus* contigs were showed in Fig.1. For gene ontology annotation, the 23,817 contigs were subjected to Blast2GO to obtain GO annotations showed in Fig.2. Total of 18,482 contigs were matched known functions after BLAST.

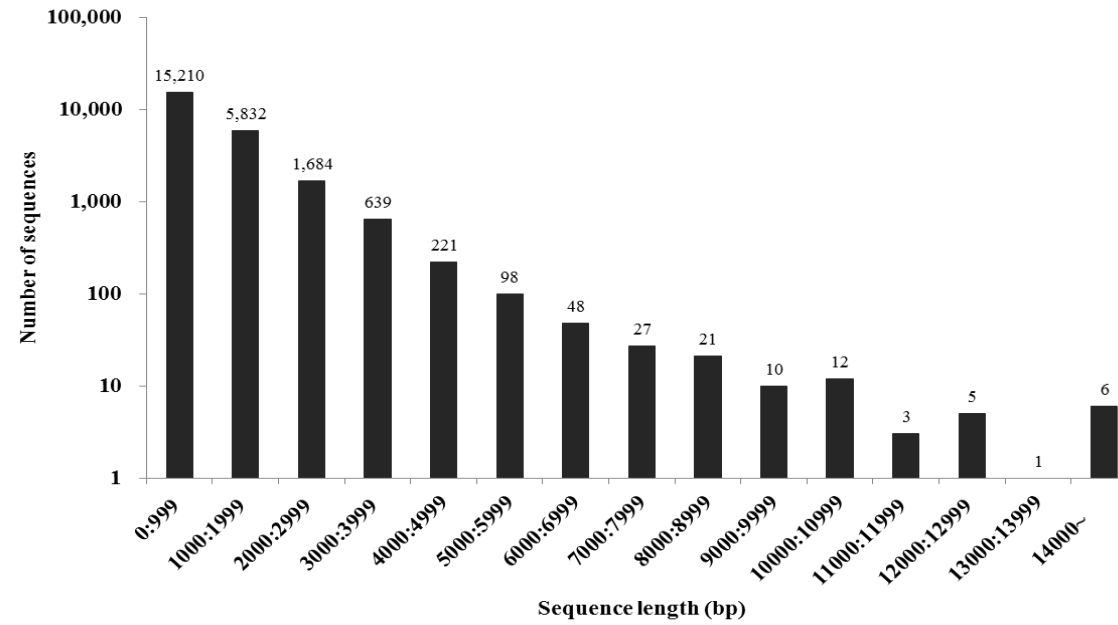


Fig.1. Length distributions of the de novo assembled cDNA library of *L. striatellus*. The length distribution of contigs was counted from 1kb to 24kb with an interval of every 1 kilobase.

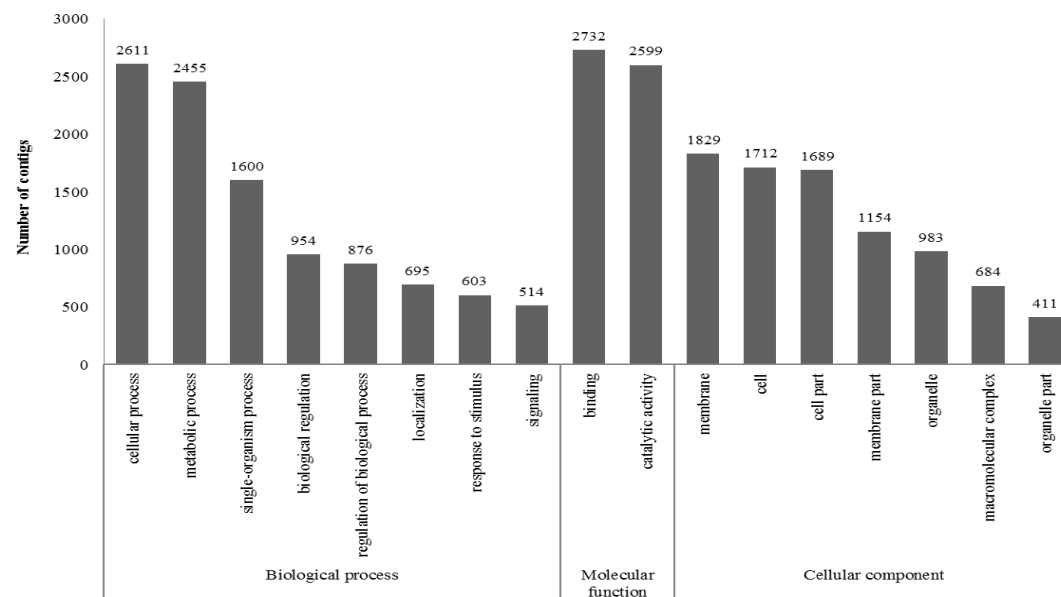


Fig.2 Gene ontology classification of *L. striatellus* cDNA library. Out of 23,817 contig sequences in the *in silico* cNDA library, total of 12,046 sequences were assigned to functional GO terms in each of the three main categories by alignment to GO terms: biological process, cellular component, and molecular function. The Y-axis indicates the number of contig sequences mapped to the GO terms

3.3 Analysis of differentially expressed genes upon buprofezin treatment

The total RNA samples were extracted from viable nymphs at 8 h post treatment, therefore, it was considered that those samples reflect the onset of sublethal dose effect of the buprofezin. The gene expression levels of each sample were quantified by mapping of the Illumina short reads to the *in silico* cDNA library by Bowtie2 program. The level of gene expression was converted to FPKM (fragments per kilobase of exon model per million fragments mapped) value by eXpress program to show 9,346 and 10,619 of the genes were down- and up-regulated under buprofezin treatment (Table 3). In the 9,346 of down-regulated genes, total of 20, 150, and 1242 genes were down-regulated over 10, 5-10, and 2-5 fold, respectively. In the 10,619 up-regulated genes, total of 19, 125, and 1515 genes were up-regulated over 10, 5-10, and 2-5 fold under buprofezin treatment, respectively. The genes which showed lower than two-fold change were considered that the expression level did not change significantly.

For GO annotation, the genes which responded to the buprofezin treatment were categorized into six groups. The first three groups were genes down-regulated over 2-5, 5-10, and 10 fold under buprofezin treatment, respectively, and other three groups were the up-regulated genes in the same way. The significantly enriched biological processes were identified as those with $FDR < 0.05$. Total five gene ontology groups which were

anatomical structure development (GO:0048856), developmental process (GO:0032502), single-organism developmental process (GO:0044767), multicellular organism development (GO:0007275) and single-multicellular organism process (GO:0044707) were highly affected by buprofezin in the up-regulated over 5-10 fold difference group. Total four gene ontology groups which were transmembrane receptor activity (GO:0099600), receptor activity (GO:0004872), molecular transducer activity (GO:0060089) and G-protein coupled receptor activity (GO:0004930) were highly affected by buprofezin in up-regulated over 2-5 fold difference group (Table 4). Moreover results showed that in up-regulated over 5-10 fold group, 5 GO terms were approximately over-enriched than reference set, 4 GO terms in 2-5 up-regulated group were approximately over-enriched than reference set (Fig.3). These results may reflect that these nine GO terms pivotally controlled by buprofezin treatment.

Table 3. Summary of differentially expressed contigs under buprofezin treatments.

Differential gene expression (Buprofezin/Control)	
Total number of contigs	23817
Total number of down-regulated genes	9346
Down-regulated over 10 fold	20
Down-regulated between 5-10 fold	150
Down-regulated between 2-5 fold	1242
Total number of up-regulated genes	10619
Up-regulated over 10 fold	19
Up-regulated between 5-10 fold	125
Up-regulated between 2-5 fold	1515

Table 4. Gene ontology enrichment analysis of differentially expressed gene groups under buprofezin treatment.

Differentially expressed gene group	Number of contigs	Differentially expressed GO terms(FDR≤0.05)
Down-regulated by buprofezin over 10- fold	20	0
Down-regulated by buprofezin over 5-10 fold	150	0
Down-regulated by buprofezin over 2-5 fold	1242	0
Up-regulated by buprofezin over 10- fold	19	0
Up-regulated by buprofezin over 5-10 fold	125	5
Up-regulated by buprofezin over 2-5 fold	1515	4

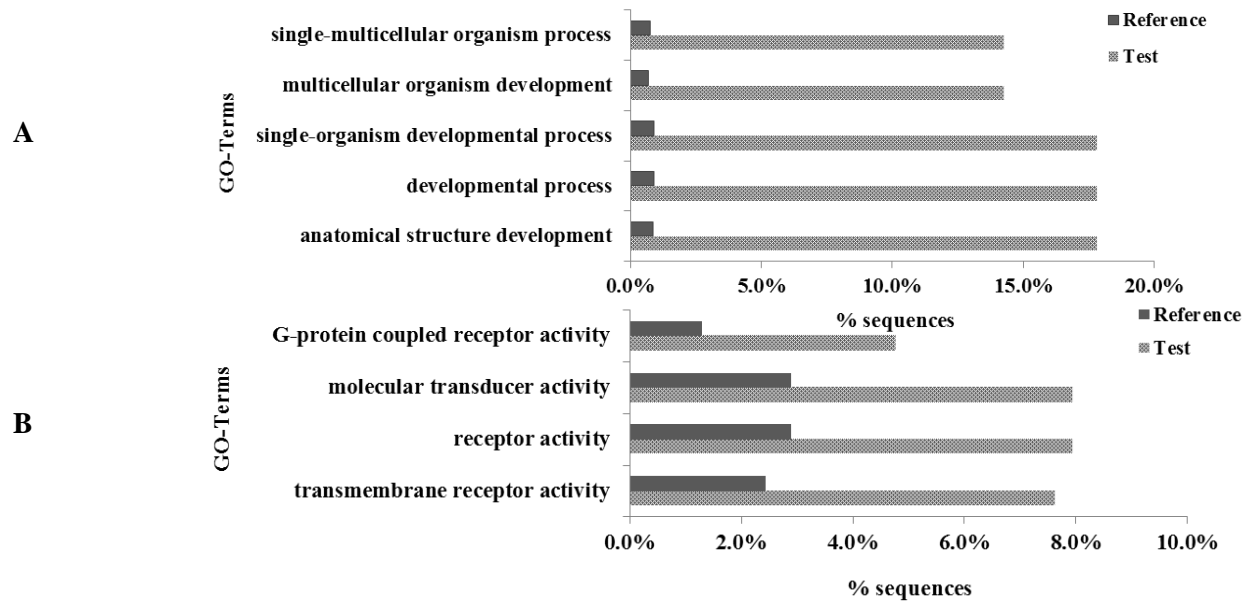


Fig.3. Functional clustering based on Gene Ontology of genes. Functional clustering based on Gene Ontology of genes that show significant differences are indicated for up-regulated over 5-10 fold differentially expressed genes under buprofezin treatment (A), and 2-5 fold differentially expressed genes (B). Percent of enriched sequences in test set and reference set were recorded respectively

3.4 Validation of the sequencing results by qPCR

Six down-regulated genes under buprofezin treatment were selected to validate the Illumina sequencing result. The transcription level of the six contigs were calculated by qPCR with $2^{-\Delta Ct}$ method, and compared with those of Illumina sequencing result in FPKM value. All qPCR results were in good accordance with the Illumina sequencing result expect the contig 59719. The qPCR result of 56615, 57447 and 57884 demonstrated that the transcription level of those three contigs were in good accordance with the sequence analysis result. Although the other three contigs including 39860, 55234 and 59719 had difference results in the value of fold difference, the down-regulated trend under buprofezin treatment was the same (Fig.4).

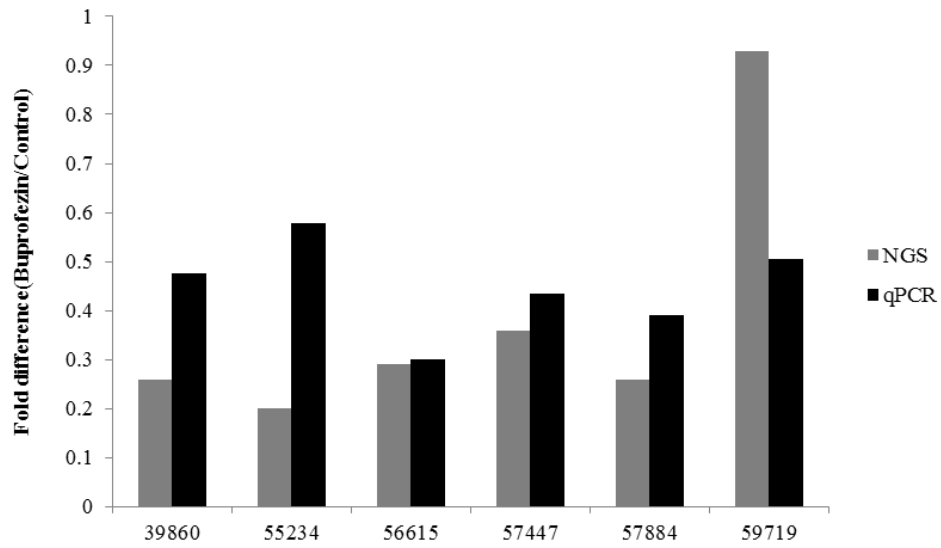


Fig.4. Validation of the Illumina sequencing result by qPCR. The transcription levels of arbitrarily selected six genes were analyzed by qPCR to verify the Illumina sequencing result. Transcription levels of the six genes were calculated by qPCR with $2^{-\Delta CT}$ method and compared with those FPKM values of Illumina sequencing, respectively. The transcription levels of each gene measured by qPCR and sequencing methods were in agreement.

4. DISSCUSSION

L. striatellus is one of the most problematic insect pests of rice in Asia. Although the ecological and physiological studies on *L. striatellus* have been extensively conducted, genomic information for *L. striatellus* is currently unavailable, and therefore, transcriptome and expression profiling data for this species are needed as an important resource to better understand the biological mechanisms of *L. striatellus*. In this study, we focused on the differentially expressed genes under buprofezin treatment and established an *in silico* cDNA library by Illumina sequencing of *L. striatellus* transcriptome which is believed to provide a valuable draft or reference gene sequences and important information for future studies of molecular biology and buprofezin-resistant mechanism of *L. striatellus*.

Although total of 194,199 contigs were initially obtained by Trinity program from the 54,150,426 of pooled, paired-end clean reads of all the samples, and the final version of *in silico* cDNA library only contains 23,817 contigs which were coding RNA genes. And the shortest and longest contigs of the final cDNA library were 297 bp and 23,796 bp, respectively. Non-coding RNAs also play the important roles in gene regulation at the level of chromatin modification, transcription and post-transcriptional processing (Mercer 2009).

The non-coding RNA sequences of *L. striatellus* were excluded from the analyses this time, however, the possible roles of non-coding RNAs need to be investigated in further study.

The previously reported *L. striatellus* cDNA database contained 41,492 unigenes and only 6,873 (16.6%) unigenes have significant matches with BLASTX search against the NCBI-NR database (Zhang 2010) while 18,482 (77.6%) contigs of our cDNA library were matched with sequences of known functions. From another study, 53,553 unigenes with a mean size of 388 bp were obtained and half of these genes had an annotation with matches in the NCBI database of *Nilaparvata lugens* (Bao 2012). The reason that other studies reported more numbers of contigs possibly that those libraries included singleton sequences while ours did not.

The Illumina sequencing is getting popular as a tool for gene expression study, and GO enrichment analysis provides a tool for interpretation of the high throughput data to expression profiles of functional set of genes. To investigate the effect of buprofezin, the genes which showed over two fold difference were categorized into six groups (Table 4). The results showed that total of nine, the majority of the differentially expressed GO terms in this study were up-regulated by buprofezin treatment (Fig.3). Among the nine GO terms, four GO terms which were anatomical structure development (GO:0048856), developmental process (GO:0032502), single-organism developmental process

(GO:0044767), multicellular organism development (GO:0007275), were related to the development function may on account of the 4th instar *L. striatellus* samples which were supposed to molt from nymph to adult, the effect of buprofezin may expresses its action at the time of molting as a chitin synthesis inhibitor (De Cock and Degheele 1998). Four molecular function GO terms which were transmembrane receptor activity (GO:0099600), receptor activity (GO:0004872), molecular transducer activity (GO:0060089) and G-protein coupled receptor activity (GO:0004930) were highly expressed in buprofezin treated *L. striatellus* than reference set that suggested buprofezin may affect the elemental activities of a gene product at the molecular level, such as binding or catalysis. Therefore the data of GO enrichment could provide a number of related contigs under buprofezin treated.

Useful and excellent agricultural pesticides have been developed in the past 50 years. However, the efficiency in finding new major compounds through biological screening has decreased. Insecticidal target molecules are mostly related to the nerve or neuron and some to the respiratory machinery or cuticle formation. Chemicals acting on other targets are quite limited. So genome studies in insects appear to contribute to finding unexplored target genes which possibly cause insecticidal activity when their expressions and functions were disrupted. Our transcriptome and gene expression profiling data greatly

enriched the current *L .striatellus* database and will contribute to research with respect to the identification of novel genes, chemical targets, developmental process and IGR pesticide resistance mechanism.

CHAPTER 2. RNA interference for insecticidal activity in Small Brown Planthopper, *Laodelphax striatellus* by using dsRNAs targeting buprofezin-specific genes

ABSTRACT

The small brown planthopper, *Laodelphax striatellus*, is one of the most serious pest insects of rice plants. buprofezin has been used to control *L. striatellus* for more than a decade; however, the occurrence of buprofezin resistant *L. striatellus* was reported recently. To develop an alternative pest control strategy, RNA-seq of buprofezin-treated *L. striatellus* was performed to screen the buprofezin-specific genes for RNA interference (RNAi). Six genes were selected for dsRNA synthesis, and applied to *L. striatellus* to assess the insecticidal efficacy. Two and three of those dsRNAs showed moderated and substantial insecticidal activity up to 60% of mortality in 7 days post treatment, respectively. These results demonstrated the potential of gene screening strategy for development of the RNAi-based pest management program.

Key words: *Laodelphax striatellus*, buprofezin, RNA-seq, RNA-interference

1. INTRODUCTION

Chemical insecticides are used worldwide for controlling agricultural insect pests. Despite the numerous benefits that could come from the usage of pesticides, the risk of environmental contamination and effects to human health remains a major concern. These chemicals can enter the environment via various routes, such as spraying activities, soil seepage and water contamination. Moreover, the persistent nature and high toxicity of these pesticides can be detrimental to public health when exposed (Kim 2017), either through consumption, dermal contact or inhalation. Also overuse of insecticides has often led to the development of resistance against most active compounds in targeted insect pests, though resistance levels tend to vary with the kind of insecticides (Roush and McKenzie 1987, Denholm and Rowland 1992, Lenormand 1999, Denholm 2002).

L. striatellus is an economically important phytophagous species in the family of Delphacidae of Hemiptera (Zhang 2010). Besides injuring rice plants (*Oryza sativa*) by sap-sucking with its piercing-sucking mouthparts, *L. striatellus* also acts as the most important vector of rice stripe virus (RSV, belonging to Tenuivirus) in a persistent and propagative manner (Ramirez and Haenni 1994). Several factors are associated with the

population dynamics of SBPH, and these include changes in the agro-ecosystem, crop rotation, climate change, and pesticide application (Yamamura 2006, Jing 2015).

Buprofezin is an IGR with activity against many insect pests. It acts by the inhibition of chitin synthesis, and has been used as insecticide for more than 20 years. As with many other chemical insecticides, widespread applications of buprofezin have resulted in high level resistance in natural populations of insect pests, such as *N. lugens* (Wang 2008) and *B. tabaci* (Cahill 1996). Also many field populations of *L. striatellus* also show resistance to buprofezin (Gao 2008, Wang 2008). In previous studies, a buprofezin-resistant strain of *L. striatellus* through many generations of selection on a susceptible line was established to investigate the mechanism underlying buprofezin resistance and found that the detoxification enzyme, cytochrome P450 monooxygenases (P450s), and NADPH-cytochrome P450 reductase (CPR) contributed to the buprofezin resistance (Zhang 2012, Zhang 2016).

RNA interference (RNAi) refers to double-stranded RNA (dsRNA) mediated gene silencing (Fire 1998). Since its discovery, it has been widely used in insect genetic research. Recently, a new hot point is to find a feasible way to use RNAi as an alternative method for practical application as insecticides (Huvenne and Smagghe 2010). In this aspect, two major technologies are considered as the most important, namely, a simple delivery system

and a high throughput screening method of optimal RNAi target genes, especially in those pests lacking any genome or transcriptome sequence information (Wang 2011). It has been reported the gene in planthoppers can be knocked down by dsRNA injection (Xue 2013), feeding of artificial diets containing dsRNA (Wan 2014) and transcribing dsRNA (Zha 2011) in planthoppers. Recent researches suggest that the NGS technologies allow directly sequencing the cDNA generated from messenger RNA (RNA-seq) (Wang 2009). This new method for analyzing RNA-seq data enables the *de novo* reconstruction of the transcriptome for a nonmodel organism (Kahvejian 2008), and also has led to novel opportunities to be used in RNAi target gene screening.

In chapter 1, the Illumina sequencing technology was employed to analyze the differential gene expression between buprofezin treated *L. striatellus* and control *L. striatellus* to select the genes down-regulated by buprofezin. Presumably, these genes are responsible to the IGR effect of buprofezin, and potential target of RNAi because down-regulation of these genes can possibly cause insecticidal activity to mimic the mechanism of buprofezin. In this study, the insecticidal activities of the selected dsRNAs targeting buprofezin-specific genes were investigated by feeding of dsRNA to *L. striatellus* through the rice plant irrigated with dsRNA solution.

2. MATERIALS AND METHODS

2.1 Selection of genes for RNAi

In chapter 1, by Illumina sequencing, we got the differential expressed genes of *L. striatellus* under buprofezin treated. Total of 57 contigs were down-regulated over two fold under buprofezin treatment and all the FPKM of these control sample contigs were over 100. Then these 57 contigs were subjected to BLAST search to NCBI database to select the candidate genes for RNAi in the next experiment.

2.2 Total RNA extraction and target gene cloning

Total RNA was extracted using Qiazol lysis reagent (Qiagen Ltd., Crawley, UK) according to the manufacturer's instructions. The procedure is the same as described in Chapter 1.

The total RNA extracted from *L. striatellus* was then used for cDNA synthesis with QuantiTect Reverse Transcription Kit (Qiagen Ltd.,Crawley, UK). Briefly, 1µg of each RNA was incubated for 2 min at 42 °C with gDNA wipeout buffer and RNase-free water to eliminate gDNA. The total RNA without gDNA then were added Quantiscript Reverse Transcriptase and RT buffer and incubated for 15min at 42 °C for activation of reverse

transcriptase. For the last step, the entire reverse-transcription reactions were incubated 95 °C for 3min to inactivate reverse transcriptase. With the synthesized cDNA, target genes were amplified with a set of primers added T7 promoter sequences (Table 5) through PCR reaction. Amplified products were visualized in 0.8% agarose gels and then ligated into pGEM-T easy vector (PROMEGA, USA) and then transformed into TOP10 competent *E.coli* cells. After 12h incubation the plasmids were purified by using QIAprep Spin Miniprep Kit (Qiagen Ltd., Crawley, UK) and confirmed by nucleotide sequencing.

2.3 dsRNA synthesis

Nucleotide sequence of the candidate genes for dsRNA treatment were applied to the BLOCK-iTTM RNAi Designer (<https://rnaidesigner.thermofisher.com/rnaiexpress>) to predict the candidate siRNA sites, and dsRNA sequences were designed to include at least three putative siRNA sites. Single-strand cDNAs of the target genes were synthesized from total RNA of the *L. striatellus* using the QuantiTect Reverse Transcription Kit (QIAGEN, Germany) according to manufacturer's instructions, and the target genes were amplified with a set of primers including T7 promoter sequence (5'-TAATACGACTCACTATAG-3') at 5'-end (Table 5). By using the amplified product as template, dsRNAs for the target

genes were produced by Genolution Pharmaceuticals (Korea). The molecular size of each synthesized dsRNA was confirmed on a 1.8% agarose gel.

Table 5. Primer information for RT-PCR of candidate dsRNA

Name	Nucleotide sequence(5'to3')	Amplicon
		Size
T7-39680-F	* T7-AGTCCCGCAACGAGCGCAACC	452bp
T7-39680-R	* T7-GACTTCACCCCAGTCATGAATC	
T7-55234-F	* T7-CAAGATGGACTACACCACTACAATT	607bp
T7-55234-R	* T7-TGAATTTGATTCAATTCTGGGCGTTG	
T7-56615-F	* T7 -TCGAGCTGTTTCATCGAGCAAGTGAA	657bp
T7-56615-R	* T7-TGAAGTTGCCGTCAACAAAGTCCTG	
T7-57447-F	* T7-GAGACACGCATTACCATTATGTAA	551bp
T7-57447-R	* T7-TAACTACTTACCCAAATTTCTCTGG	
T7-57884-F	* T7-CCATTGTCCACAAGGAGCCTGAGAT	628bp
T7-57884-R	* T7-TTAACCAGCCATGTGCCGTGATAGG	
T7-59719-F	* T7-TCGAACATCTGATAACCATAC	550bp
T7-59719-R	* T7-CTCAGAAATATTTGAGAGTAG	

* T7 sequence: TAATACGACTCACTATAG

2.4 Delivery of dsRNA to *L. striatellus* and Insect bioassay

Fifteen 4th instar nymphs of *L. striatellus* were ingested with dsRNA by using rice-mediated feeding RNAi method according to previously report described (An 2017). Briefly, a feeding chamber was fabricated by assembling 15-ml conical tube (Fisher Scientific, USA) with the hole on top of the tube to provide air and a 10- μ l tip was placed into the hole to prevent *L. striatellus* from escaping the tube. Parafilm Mfilm (Bemis, USA) was placed to seal gap between 15-ml tube and tube cap consisting feeding chamber to make a reservoir for holding dsRNA solution.

For screening the mortality of *L. striatellus* under buprofezin-specific dsRNA treatment, the six dsRNAs with 250 ng/ μ l of final concentration in 10% of sucrose solution were respectively delivered to nymphs. To investigate the combinational mortality effects of buprofezin-specific dsRNAs, the dsRNA cocktails containing all of the candidate dsRNAs or except one each designated minus with equal 250 ng/ μ l final concentration were respectively delivered to nymphs. To measure the dose-dependent mortality of *L. striatellus* under dsRNA treatment, 30, 60, 120, 180 and 250 ng/ μ l concentration of each dsRNA or dsRNA cocktails were treated to nymphs. The dsRNA-treated rice leaves infested with *L. striatellus* in the feeding chamber were placed in growth chamber at 28 °C,

80% relative humidity, and 16:8h (light:dark) photoperiod. All insecticidal dsRNA tests were observed for 7 days persistently.

To examine synergistic insecticidal activity of buprofezin-specific dsRNA and buprofezin, 0.2, 2, 5, 10, 20 and 200 ppm buprofezin was respectively dipped to *L. striatellus* reared on the healthy rice, the mortality was recorded at 72 h post treatment. On other side, the same buprofezin treated *L. striatellus* were reared on the dsRNA treated rice and mortality was recorded at 72 h post treatment. Mortality studies consisting of two biological replicates and two technical replicates each was analyzed with the program package SPSS (SPSS, 2001). Scheffe's post hoc tests were used to identify groups that differed. Effects were considered significant at the $P < 0.05$ level.

2.5 Quantitative PCR analysis of knock down

The *L. striatellus* fed on dsRNA-treated rice leaves were collected after 48h for RNA extraction using the same method mentioned before. Complementary DNA was synthesized with oligo-d(T) primer using QuantiTect Reverse Transcription Kit (QIAGEN, Germany). Quantitative PCR was conducted using the EvaGreen qPCR Master Mix (Applied Biological Materials, Canada) and a CFX96TM Real-Time System (BIO-RAD, USA) according to the manufacturer's instructions. The cycling profile used for qPCR was

as follows: a preheating step for enzyme activation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 sec, 55 °C for 15 sec and 72 °C for 30 sec. The ADP ribosylation factor (ARF) was used as a reference gene. The relative transcription levels were calculated using the $2^{-\Delta C_t}$ method (Pfaffl 2001). The primers used for qPCR are listed in Table 6.

Table 6. Primer used in qPCR

Gene name	Target-ID	Forward primer	Reverse Primer
39860	comp39860_c0_seq2	CTTACGACCAGGGCTACACA	ACTCCAATCCGGACTACGAC
55234	comp55234_c0_seq2	GCTTACACATTCTTCAGCCT	TGAAATGGAAGGTTGGTGTG
56615	comp56615_c0_seq1	AGGTGGTGTGGGTAGGAAAC	TTCAGTTTGGGACTGTCAGC
57447	comp57447_c0_seq6	TTCCCATTTGGACCAGCTT	ACTTCAGTTGTCCACAAGTATCG
57884	comp57884_c0_seq7	AGTACCAGGAGACAGACGCA	TTTCCCTTGCGAATAACCTC
59719	comp59719_c0_seq1	AGAGTCGAACATCTGATGATACCATAC	AGAGCTCAGAAATATTTGAGAGTAG
ARF	ADP-ribosylation	TTGGACAGTATCAAGACCCATC	GCAGCAATGTCATCAATAAGC

3. RESULT

3.1 Selection of genes for RNAi

Considering the FPKM value and the down-regulation under buprofezin, the information of top 20 genes which have lowest ratio of buprofezin to control were listed in Table 7. According to the ratio of buprofezin and Control, five buprofezin-specific contigs with the minimum ratio were selected to synthesize dsRNA. Because buprofezin is a chitin synthesis inhibitor insecticide, comp59719 predicted code chitin synthase was also selected to synthesize dsRNA. And these six selected buprofezin-specific contigs were renamed to 39860, 55234, 56615, 57447, 57884 and 59719 listed in the Table 8.

Table 7. The list of down-regulated genes upon buprofezin

treatment.

	Target id	Length (bp)	Buprofezin (fpkm)	Control (fpkm)	B/C	Gene Function
1	comp55234_c0_seq2	2091	47.25	230.88	0.20	<i>Laodelphax striatella</i> clone 1 cytochrome P450 CYP4C62 mRNA
2	comp39860_c0_seq2	411	1115.57	4236.08	0.26	<i>Enterobacter sp.16S</i> ribosomal RNA gene
3	comp57884_c0_seq7	2584	35.02	132.90	0.26	<i>Drosophila takahashii</i> acyl-CoA Delta(11) desaturase
4	comp56615_c0_seq1	11220	30.36	103.84	0.29	<i>Nilaparvata lugens</i> mRNA for lipophorin precursor
5	comp46138_c0_seq1	788	147.51	481.99	0.31	<i>Linepithema humile</i> prefoldin subunit 6
6	comp57447_c0_seq6	1437	51.21	141.73	0.36	<i>Tribolium castaneum</i> endocuticle structural glycoprotein SgAbd-8
7	comp44458_c0_seq1	689	48.21	120.40	0.40	<i>Polistes dominula</i> CDGSH iron-sulfur domain-containing protein 3
8	comp52532_c0_seq1	1014	113.29	276.80	0.41	<i>Halyomorpha halys</i> prefoldin subunit 1
9	comp21924_c0_seq1	348	67.19	162.56	0.41	<i>Drosophila melanogaster</i> SRP RNA for signal recognition particle RNA
10	comp31277_c0_seq1	744	46.60	110.04	0.42	<i>Microplitis demolitor</i> chromatin accessibility complex protein 1-like
11	comp47341_c0_seq1	850	261.39	592.93	0.44	<i>Nilaparvata lugens</i> defensin B mRNA
12	comp53510_c0_seq3	1813	91.26	205.54	0.44	<i>Cerapachys biroi</i> inositol-3-phosphate synthase 1-B
13	comp47354_c0_seq1	1695	72.80	158.26	0.46	<i>Bombus terrestris</i> ribonuclease UK114 (LOC100646186), mRNA
14	comp56523_c0_seq1	2653	86.21	184.83	0.47	<i>Apis mellifera</i> digestive cysteine proteinase 1
15	comp46173_c0_seq1	577	146.31	313.04	0.47	<i>Nasonia vitripennis</i> signal recognition particle 14 kDa protein
16	comp49057_c0_seq1	2472	59.44	126.66	0.47	<i>Laodelphax striatella</i> putative asparagine synthetase isoform 2 mRNA
17	comp56730_c0_seq1	3108	102.87	218.45	0.47	<i>Diaphorina citri</i> carbonic anhydrase 13
18	comp39782_c0_seq1	576	443.11	935.95	0.47	<i>Bombyx mori</i> ribosomal protein S11
19	comp45905_c0_seq1	2057	239.11	503.69	0.47	<i>Drosophila elegans</i> fructose-bisphosphate aldolase
20	comp47835_c0_seq2	3328	241.86	509.39	0.47	<i>Nilaparvata lugens</i> alpha-tubulin 5 mRNA

Table 8. The list of selected buprofezin-specific genes upon buprofezin treatment.

Contig name	Rename	Length (bp)	Buprofezin (fpkm)	Control (fpkm)	B/C	Gene Function
comp39860_c0_seq2	39860	411	1115.57	4236.08	0.26	<i>Enterobacter</i> sp.16S ribosomal RNA gene
comp55234_c0_seq2	55234	2091	47.25	230.88	0.2	<i>Laodelphax striatella</i> clone 1 cytochrome P450 CYP4C62 mRNA
comp56615_c0_seq1	56615	11220	30.36	103.84	0.29	<i>Nilaparvata lugens</i> mRNA for lipophorin precursor
comp57447_c0_seq6	57447	1437	51.21	141.73	0.36	<i>Tribolium castaneum</i> endocuticle structural glycoprotein SgAbd-8
comp57884_c0_seq7	57884	2584	35.02	132.9	0.26	<i>Drosophila takahashii</i> acyl-CoA Delta(11) desaturase
comp59719_c0_seq1	59719	6892	19.57	20.9	0.93	Chitin synthase

3.2 The confirmation of synthesized dsRNA

The synthesized dsRNA of 39680, 55234, 56615, 57447, 57884 and 59719 were confirmed on a 1.8% agarose gel. ds39680, ds55234, ds56615, ds57447, ds57884 and ds59719 yielded expected product of 452bp, 607bp, 657bp, 551bp, 628bp and 550bp, respectively. As a result of this gel electrophoresis, the synthesized dsRNA were confirmed (Fig.5).

3.3 Silencing of candidate genes in *L. striatellus* ingested buprofezin-specific dsRNAs

By feeding RNAi system, silencing of the six selected genes upon Burprofezin-specific dsRNAs treated and dsGFP treated *L. striatellus* were confirmed by using qPCR. As show in Fig.6, the expression of six selected genes was all decreased at 48 h under 250 ng/μl Burprofezin-specific dsRNA treatments. Four of buprofezin-specific genes, 55234, 57447, 57884 and 59719, their relative transcription levels were reduced over 50% compared to those of dsGFP treatment. The other two genes, 39860 and 56615, their relative transcription levels were only reduced to 30% compared to dsGFP treatment. Although the value of FPKM was related to the gene transcription level, not all the genes showed the same trend. The FPKM of gene56615 was not the highest, but the related

transcription level was the highest. The FPKM ratio of gene59719 was 0.93, but the relative transcription level was reduced over 50% under ds59719 treatment.

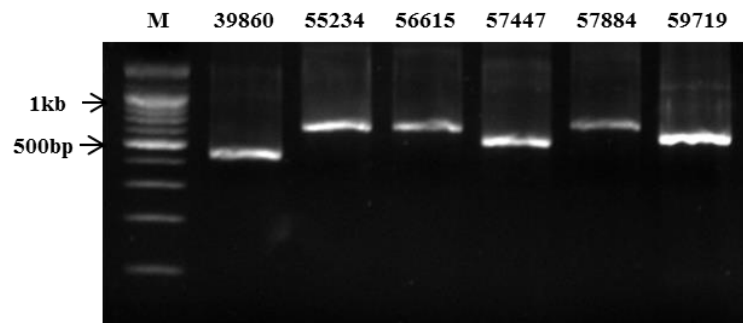


Fig. 5. Agarose gel confirmation of synthesized dsRNA. ds39680, ds55234, ds56615, ds57447, ds57884 and ds59719 yielded expected product of 452bp, 607bp, 657bp, 551bp, 628bp and 550bp. M, 1kb DNA ladder.

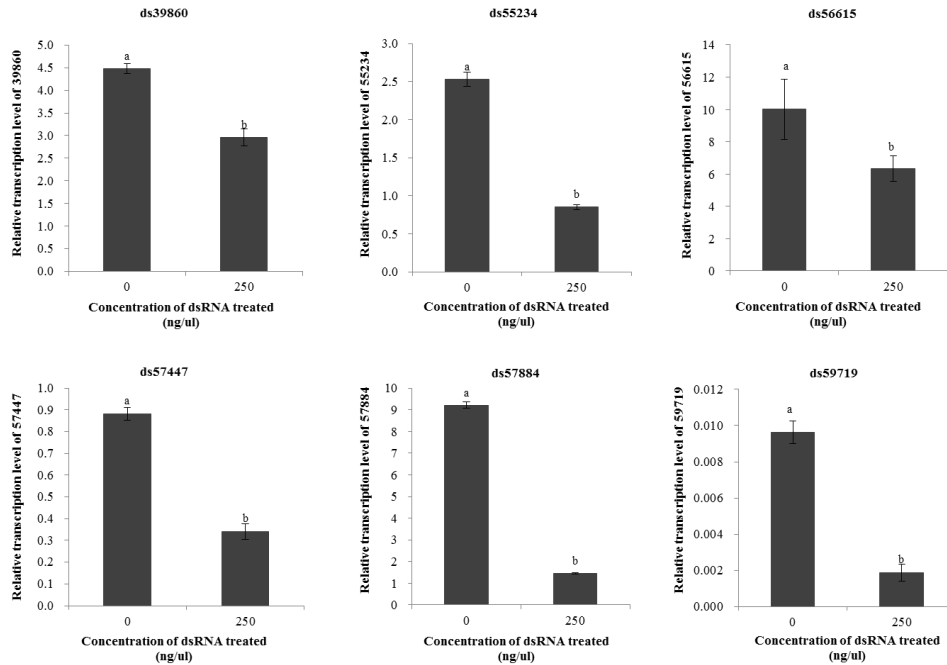


Fig. 6. Silencing of candidate genes in *L. striatellus* ingested buprofezin-specific dsRNAs. Fifteen 4th instar nymphs of *L. striatellus* were fed on rice leaves steeped in dsGFP (Genolution Pharmaceuticals, Korea) or six buprofezin-specific dsRNAs for 48 h. Relative transcription levels of six buprofezin-specific genes in *L. striatellus* were measured by qPCR. All assays were performed in triplicates and different letters above error bars (indicating \pm standard deviations) indicate a significant difference by Scheffe's post hoc tests ($P < 0.05$)

3.4 Silencing of buprofezin-specific genes by dsRNA causing increased mortality

Six dsRNAs targeting buprofezin-specific genes were supplied *ad libitum* to *L. striatellus* for seven days. The *L. striatellus* treatment with ds39860 did not show significant difference of mortality compared to that of dsGFP or negative control. However, the other five dsRNAs showed significant impact on nymphal mortality. Especially, ds56615, ds57447 and ds59719 showed significantly higher mortality than the others at the same concentrations of 250 ng/μl (Fig.7). Moreover, the *L. striatellus* treated with dsRNA cocktails containing all five of ds55234, ds56615, ds57447, ds57884 and ds59719 or four of those except one each designated minus with the equal 250 ng/μl final concentration showed that ds56615, ds57447 and ds59719 had higher mortality than ds55234 and ds57884 (Fig.8). Then dsRNA cocktails containing all three of the selected -ds56615, ds57447 and ds59719 or two of those except one each designated minus with the equal 250 ng/μl final concentration were delivered to *L. striatellus*, and the mortality results did not show significant differences between these three dsRNA treatment (Fig. 9).

To confirm the dose-dependent mortality of *L. striatellus* under ds56615, ds57447 and ds59719 treatment, 30, 60, 120 and 180 ng/μl concentration dsRNAs were treated to *L. striatellus*. The result showed the mortality was increased with higher concentration of

dsRNA treatment and especially ds56615 of 180 ng/μl concentration caused over 40% mortality higher than the other two dsRNAs (Fig. 10). Also the dsRNA cocktail containing ds56615, ds57447 and ds59719 was supposed to have dose-dependent mortality of *L. striatellus* from 0 to 250 ng/μl concentration treatments (Fig. 11), and there was no significant difference between 180 ng/μl and 250 ng/μl dsRNA treatment.

Synergistic insecticidal activity of buprofezin-specific dsRNAs and buprofezin were also surveyed. buprofezin insecticide experiment showed *L. striatellus* reached 70% mortality when treated with 200 ppm and only 40% mortality under 5 ppm chemical treatments (Fig.12). However, the mortalities of *L. striatellus* pretreated with low concentrations of buprofezin followed by feeding of dsRNA cocktail were up to 80%. Treatment of 0.2ppm buprofezin did not show insecticidal effect to *L. striatellus*, however, treatment with dsRNA cocktail containing buprofezin-specific dsRNAs was able to cause approximately 30% of mortality (Fig. 13). Only the treatment with 5 ppm buprofezin and dsRNA cocktail was able to reach 80% mortality, which was 40% higher than that of buprofezin only treatment.

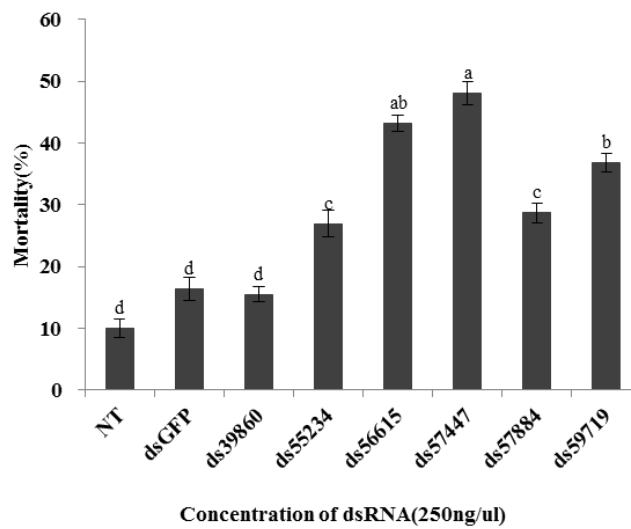


Fig. 7. The mortality of *L. striatellus* at 7 days post treatment of single buprofezin-specific dsRNA treatment. Each group of fourth instar nymphs was continuously supplied *ad libitum* with rice seedlings irrigated with 250 ng/ μ l of each dsRNA. All assays were performed in triplicates and different letters above error bars (indicating \pm standard deviations) indicated a significant difference by Scheffe's post hoc tests ($P < 0.05$). (NT= Negative control)

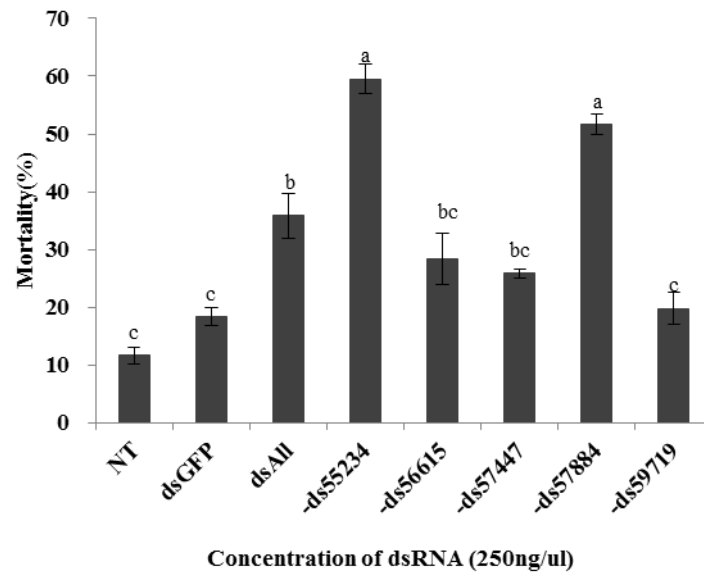


Fig. 8. The mortality of *L. striatellus* at 7 days post treatment of dsRNA cocktail containing five buprofezin-specific dsRNA. Each group of 4th instar nymphs were continuously supplied *ad libitum* with rice seedlings irrigated with 250 ng/μl of dsRNA cocktail. The dsRNA cocktails containing all of the five dsRNAs or four dsRNAs except one each designated with minus sign under equal concentration. All assays were performed in triplicates and different letters above error bars (indicating \pm standard deviations) indicated a significant difference by Scheffe's post hoc tests ($P < 0.05$). (NT= Negative control)

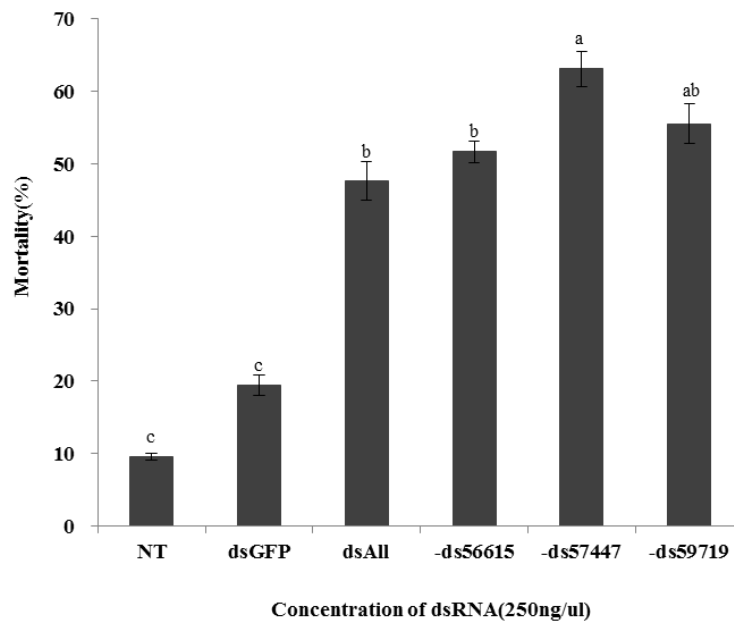


Fig. 9. The mortality of *L. striatellus* at 7 days post treatment of dsRNA cocktail containing three buprofezin-specific dsRNA. Each group of fourth instar nymphs were continuously supplied *ad libitum* with rice seedlings irrigated with 250 ng/μl of dsRNA cocktail. The dsRNA cocktails containing all of the three dsRNAs or two dsRNAs except one each designated with minus sign under equal concentration. All assays were performed in triplicates and different letters above error bars (indicating \pm standard deviations) indicated a significant difference by Scheffe's post hoc tests ($P < 0.05$). (NT= Negative control)

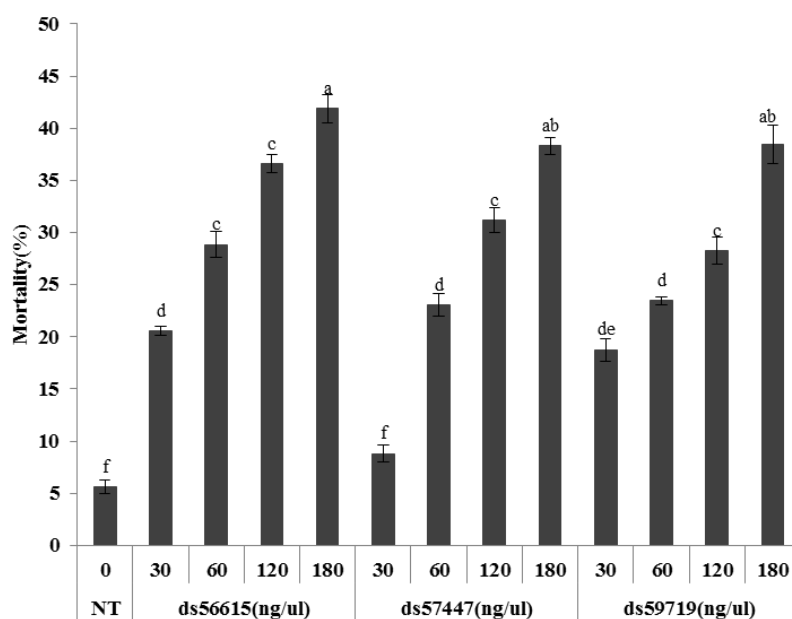


Fig. 10. The mortality of *L. striatellus* at 7 days post treatment of dose-dependent buprofezin-specific dsRNA treatment. Each group of fourth instar nymphs was continuously supplied *ad libitum* with rice seedlings irrigated with dose-dependent dsRNA. All assays were performed in triplicates and different letters above error bars (indicating \pm standard deviations) indicated a significant difference by Scheffe's post hoc tests ($P < 0.05$). (NT= Negative control)

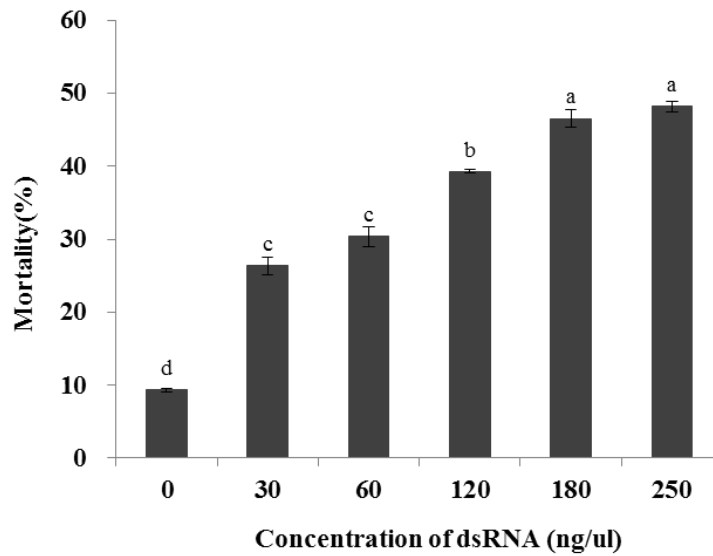


Fig. 11. The mortality of *L. striatellus* at 7 days post treatment of dose-dependent buprofezin-specific dsRNA cocktail treatment. Each group of fourth instar nymphs was continuously supplied ad libitum with rice seedlings irrigated with dose-dependent dsRNA cocktail containing ds56615, ds57447 and ds59719. All assays were performed in triplicates and different letters above error bars (indicating \pm standard deviations) indicated a significant difference by Scheffe's post hoc tests ($P < 0.05$).

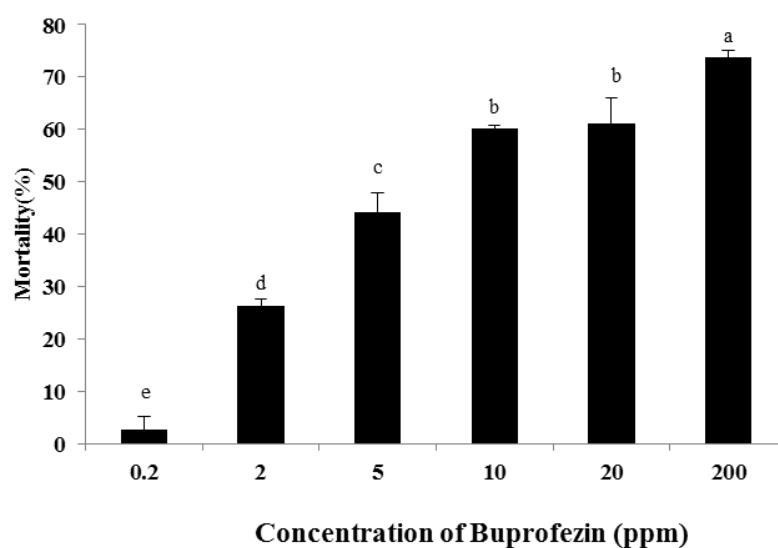


Fig. 12. The mortality of *L. striatellus* at 72 h treatment upon dose-dependent concentration buprofezin by dipping method. All assays were performed in triplicates and different letters above error bars (indicating \pm standard deviations) indicated a significant difference by Scheffe's post hoc tests ($P < 0.05$).

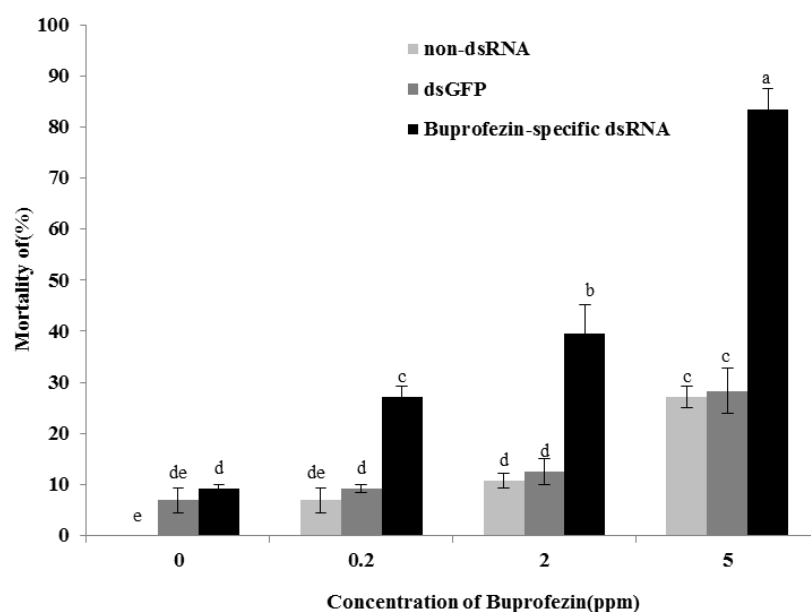


Fig. 13. The mortality of the *L. striatellus* at 72 h treatment under buprofezin-specific dsRNA cocktail treatment after buprofezin dipping treated previously. The nymphs were dipped with buprofezin solution of different concentration by 30 seconds, and then reared on the rice seedlings which were cultured in buprofezin-specific dsRNA cocktail or dsGFP for negative control at the same concentration of 250 ng/μl. All assays were performed in triplicates and different letters above error bars (indicating \pm standard deviations) indicated a significant difference by Scheffe's post hoc tests ($P < 0.05$).

4. DISSCUSSION

Taking advantage of known genes is a simple and effective way to verify RNAi targets, but the scope of selection was limited (Bautista 2009, Whyard 2009). RNA-seq is a high throughput sequencing technology which does not include any cloning or amplification step, and requires less RNA sample, and is not limited to detecting transcripts corresponding to existing genomic sequences, thus it is especially suitable for non-model organisms (Wang 2009). Therefore, we chose this method to select down-regulated target genes under buprofezin treatment for RNAi test to explore potential insecticidal dsRNA in this study. Using this method, among the 23817 contigs, 9346 and 10619 genes were down- and up- regulated upon buprofezin treatment, respectively. After validation, we synthesized six candidate dsRNAs to perform RNAi experiment. They were bacterial 16S ribosomal RNA gene of gene39860, cytochrome P450 of gene55234, lipophorin precursor of gene56615, endocuticle structural glycoprotein, acyl-CoA desaturase and Chitin synthase.

Although deep 16S rRNA sequencing was not been analyzed in our experiment, gene39680, 16S rRNA was found in differential gene expression analysis. Because any RNA fragments containing span exon junctions or polyA sequences could be mapped in the same way in RNA-seq process (Wang 2009), and the ratio of gene 39680 reads in

whole transcriptome sequencing was very low, so it is possible to find the bacterial 16S rRNA gene in the cDNA library. Generally, the 16S rRNA gene has been used as a molecular marker enabling the detection of as-yet-uncultured microbes (Weisburg 1991, Haynes 2003). Symbionts have been found to contribute to the nutrition, development, reproduction, speciation, and defense against natural enemies of their host insects (Baumann 2005). There have been reported that the small brown planthopper had the diversity of the bacterial microbes and they may associated with different populations and specific tissues (Tang 2010). In our experiment, 16S rRNA gene 39860 was down-regulated after buprofezin treatment, but ds39860 did not show significant mortality to *L. striatellus*. This result showed that buprofezin might have effect to symbiotic microbes in *L. striatellus*, but silencing of the gene39860 had no effect to survival of *L. striatellus*. Silencing of other five genes 55234, 56615, 57447, 57884, and 59719 all caused increased mortality to *L. striatellus*, and the RNAi experiment using dsRNA cocktail demonstrated that ds56615, ds57447, and ds59719 had more significant mortality effect to *L. striatellus*.

Gene 56615 indicated lipophorin precursor of insects. Lipophorin is the major hemolymph lipoprotein, and its main function is to transport lipids throughout the insect body, loading dietary lipids in the midgut and delivering them to the sites of their

metabolism and storage. In the mosquito, *Aedes aegypti*, lipophorin was accumulated as a yolk protein precursor (Sun 2000). But there have been few studies about lipophorin in planthoppers. Silencing of gene 56615 caused decrease synthesis of lipophorin to affect the transportation of lipids and possibly resulted in low survival in *L. striatellus*.

Endocuticle structural glycoprotein was isolated from the endocuticles of the migratory locust *Locusta migratoria* and the desert locust *Schistocerca gregaria*, but little is known about its function (Jespersen 1994). In our study, silencing of gene 57447 caused the increase in mortality of SBPH, suggesting that endocuticle structural glycoprotein plays an important role in synthesis of which was reported in case of *Locusta migratoria* (Zhao 2017).

Chitin synthase coded by gene 59719 was studied intensively in planthoppers. The chitin biosynthesis pathway is highly conserved, and the principal enzyme involved in this pathway is chitin synthase (CHS), which is responsible for the last step of chitin polymer formation (Candy and Kilby 1962). Two types of CHS have been observed in most insects: CHSA and CHSB. CHSA is responsible for chitin synthesis in the cuticle, trachea, eggs and ovaries, whereas CHSB has been observed in insect midgut epithelial cells in the peritrophic matrix (PM) (Merzendorfer 2006). The RNAi of NICHSA has been shown to result in a high mortality and moulting defects in *N. lugens* nymphs (Wang 2012). Also, in

our experiment silencing of gene59719 caused significant mortality to *L. striatellus* and possible reason might be the decreased transcription of chitin synthase which might affect molting.

Among the major enzyme Cytochrome P450 monooxygenases (P450s) was involved in conferring resistance to many types of insecticides, and they play roles in the detoxification and bioactivation of insecticides in various insecticide-resistant insects, and reported, in *N. lugens*, the mosquito *Culex pipiens quinquefasciatus*, and the greenhouse whitefly *Trialeurodes vaporariorum* (Westwood) (Hardstone 2010, Bass 2011, Karatolos 2012). In *L. striatellus*, an increase in P450 activity was also found in buprofezin-resistant *L. striatellus*, and one of the 38 P450 genes in *L. striatellus* was highly overexpressed in the buprofezin-resistant strain compared with the susceptible strain (Zhang 2012). In our experiment, cytochrome P450 gene55234 was down-regulated under buprofezin treatment and also silencing of gene55234 caused reduced mortality to SBPH but it was not significant. Gene57884 was 87% similar with the *Nilaparvata lugens* acyl-CoA Delta(11) desaturase-like mRNA. Little is known about acyl-CoA desaturase in planthoppers. And gene expression level of fatty acyl-CoA reductase was significantly repressed at high temperature that showed to play an essential role in low and high temperatures in planthopper species (Huang 2017). Because of our experiment was performed in a

controlled laboratory condition, silencing of gene57884 did not show the significant decrease mortality in the SBPH.

Buprofezin is considered to have low acute toxicity to humans and other mammals and has been used widely in integrated pest management programs results in the development of resistance in planthoppers. However, recent research indicated that buprofezin could perturb the energy metabolism of mouse liver (Ji 2016). Buprofezin residue can be easily absorbed by the human body via the oral cavity, the skin and the respiratory tract (Culleres 2007) and therefore has potentially adverse effects on human health. Our experiment provides a new point of view of using this effectual insecticide. Because RNAi was recognized as a novel and safe strategy in the fight against important pest organisms (Huvenne and Smagghe 2010), buprofezin-specific genes found in our experiment could be used control against *L. striatellus*.

In conclusion, our results demonstrate that a direct delivery via host plant is a feasible way to introduce dsRNA into the SBPH, which then leads to a down-regulation of gene expressions and finally to death of *L. striatellus*. The three target genes of 56615, 57447 and 59719 could be utilized for the development of pest-resistant transgenic plants based on RNAi. However, some questions still remain to be further investigation, such as in which tissues or cells where these genes are silenced and whether the proposed receptor

mediated endocytosis and RNAi is relatively complex and premature in terms of practical application in pest insect control, so more research should be explored before dsRNA can be used as insecticides. Nevertheless, our findings considerably broaden the selection of targets for RNAi research from insecticide treatment insects. It may lead to new strategies in designing the RNAi-based technology against insect damage.

CHAPTER 3. Silencing of rice stripe virus in *Laodelphax striatellus*

using SBPH-derived double-stranded RNAs

Abstract

Rice stripe virus (RSV) is one of the serious plant pathogenic viruses for rice transmitted by small brown planthopper (SBPH), *Laodelphax striatellus*. RNA interference (RNAi) was recently suggested as a promising strategy for controlling insect pests, including those that serve as important vectors for plant pathogens. To disturb transmission of the RSV in SBPH by using RNAi pathway, we chose nine genes highly expressed in RSV-viruliferous SBPH by transcriptome sequencing. These SBPH-derived dsRNAs were applied to the insects indirectly through xylem of rice leaves by irrigation. qPCR result demonstrated that three out of eight SBPH-derived dsRNAs successfully reduced the replication of RSV in viruliferous SBPH in dose-dependent manner, suggesting that these four dsRNAs could suppress replication of RSV and provide a new tool for RSV control strategy.

Key words: *Laodelphax striatellus*, Rice stripe virus, RNA interference,
double-stranded RNA

1. Introduction

The small brown planthopper, *Laodelphax striatellus* scattered all around the world is one of the sucking insect pests and transmits the rice stripe virus (RSV) in a circulative-propagative manner (Toriyama 1986). Once *L. striatellus* ingests RSV by feeding on infected plants, the virus initially infects the midgut epithelium, and then spreads to other tissues and organs through hemolymph (Hogenhout 2008). Ultimately, RSV invades into the salivary glands and reproductive system (Wu 2014). For successful transmission of plant viruses in their insect vectors, specific interactions between viral proteins and vector-associated compounds is required (Power 2000).

Rice stripe virus (RSV), a single-stranded RNA virus of the genus Tenuivirus, causes rice stripe disease, which is one of the most notorious rice diseases in temperate and subtropical regions (Toriyama 1986, Falk and Tsai 1998). RSV uses a negative-sense and ambisense coding strategies to encode seven proteins, which are RNA-dependent RNA polymerase (RdRp), NS2, NSvc2 (putative membrane glycoprotein), NS3 (gene silencing suppressor), Cp (nucleocapsid protein), Sp (nonstructural disease-specific protein) and NSvc4 (movement protein) (Hayano 1990, Zhu 1992, Takahashi 1993, Toriyama 1994, Xiong 2008). Typical symptoms of RSV include pale and discontinuous yellow stripes, blotches and dead tissue streaks on the leaves.

RNA interference (RNAi) is a post-transcriptional gene regulation mechanism in eukaryotes (Ding 2010, Merkling and van Rij 2013). The mechanism of RNAi involves the cleavage of double stranded RNA (dsRNA) by the RNase III, Dicer, into 21–25 nt-long small interfering RNA duplexes (siRNA) in an ATP dependent manner (Bass 2000). These siRNAs interact with Argonaute proteins to form the multi-protein RNA-induced silencing complex (RISC), which recognizes and degrades complementary mRNA in a sequence-specific manner (Hammond 2005). RNAi has been considered as a promising strategy for silencing of plant viruses in their insect vectors including planthoppers, aphids, whiteflies, and thrips (Kanakala and Ghanim 2016). In these approaches, target genes of insect vectors could be silenced not only for inducing mortality of insect vector itself but also for interfering with the transmission of plant viruses (Kanakala and Ghanim 2016). In *L. striatellus*, it was reported that RNAi-based silencing of cuticular protein, CRP1, transcription decreased transmission efficiency of RSV (Liu 2015).

Recently, siRNAs has been identified in *L. striatellus* infected with RSV through small RNA deep sequencing, demonstrating the potential existence of RNAi-mediated immunity against RSV in *L. striatellus* (Xu 2012, Li 2013). Feeding dsRNA via artificial saps or transgenic plant was evaluated for major hemipteran sucking insects such as whitefly (Upadhyay 2011), aphids (Pitino 2011) and leafhoppers (Chen 2010, Li 2011, Zha 2011).

By transcriptome surveys of RSV-viruliferous *L. striatellus*, we identified highly expressed genes and selected the related genes with RSV of *L. striatellus* from previously published papers (Zhao 2016), and investigated expression profile of these genes in RSV-viruliferous and non-viruliferous *L. striatellus*. Then we used an RNAi technique based on dsRNA delivery via leaf to knock-down the target genes to figure out the possibility of applying dsRNA to control rice stripe virus.

2. Materials and methods

2.1 Insect culture and inoculation of RSV to non-viruliferous SBPH

As the previous chapter, the non-viruliferous *L. striatellus* were collected from the healthy rice (*Oriza sativa*) field and reared on uninfected rice in the insectary at 28 °C, 80% relative humidity, and 16:8 (light:dark) photoperiod. To obtain RSV-viruliferous *L. striatellus*, 2nd instar nymphs of the non-viruliferous *L. striatellus* were fed on 5-6cm tall RSV-infected rice for 24h, 72h and 7 days in the insect growth chamber (28°C, 80% RH, 16:8 (L:D) photo period).

2.2 Target gene design from SBPH genes

The nucleotide sequence of 11 target SBPH genes which were up-regulated in RSV infected SBPH were obtained from the transcriptome analysis of SBPH carrying RSV. And 7 SBPH genes related to RSV which have been reported previously were also selected to proceed. The transcription level of these 18 candidate genes in RSV-viruliferous and non-viruliferous SBPH were screened by qPCR. Then selected target SBPH genes were applied to the Invitrogen BLOCK-iT™ RNAi Designer software to find the candidate

siRNA site. Each dsRNA for the target gene was designed to include at least three putative siRNA sites.

2.3 Total RNA extraction and target gene cloning

The total RNA extracted from RSV-viruliferous SBPH was used for cDNA synthesis with QuantiTect Reverse Transcription Kit (Qiagen Ltd., Crawley, UK). The procedure is the same as described in Chapter 1. The target gene cloning is also same as described in Chapter 2. Briefly, 1 µg of each RNA was incubated for 2 min at 42 °C with gDNA wipeout buffer and RNase-free water to eliminate gDNA. The total RNA without gDNA then were added Quantiscript Reverse Transcriptase and RT buffer and incubated for 15min at 42 °C for activation of reverse transcriptase. For the last step, the entire reverse-transcription reactions were incubated 95 °C for 3min to inactivate reverse transcriptase. With the synthesized cDNA, the target genes were amplified with a set of primers added T7 promoter sequences (Table 9) through PCR reaction. The amplified amplicon were ligated into pGEM-T easy vector (PROMEGA, USA) and then transformed into TOP10 competent *E.coli* cells. After 12h incubation the plasmids were purified by using QIAprep Spin Miniprep Kit (Qiagen Ltd.,Crawley, UK) and confirmed by nucleotide sequencing.

Table 9. Primer information of candidate dsRNA reducing RSV.

Amplicon	Name	Nucleotide sequence(5'to3')	Amplicon Size
75761 of <i>L.striatellus</i>	T7-75761-F	T7 promoter sequence+ GGCGTTTGTGCTGCTATGTGTTGCA	620bp
	T7-75761-R	T7promotersequence+ TGAAATTTCTGGTAGTATTGTGCGC	
76485 of <i>L.striatellus</i>	T7-76485-F	T7 promotersequence+ GAGAAGATGACACAGATCATGTTTG	672bp
	T7-76485-R	T7 promoter sequence+ TCATGATGGAGTTGTAGACGGTCTC	
78749 of <i>L.striatellus</i>	T7-78749-F	T7 promoter sequence+ GTCCTCCATCAGAAAGATCGTTC	601bp
	T7-78749-R	T7 promoter sequence+ AAAGTGAGCCCTCAAAGAATGTCCG	
80690 of <i>L.striatellus</i>	T7-80690-F	T7 promoter sequence+ GGGCATATTGATGTCTCGTTATAAA	585bp
	T7-80690-R	T7 promoter sequence+ TAATAAGCCTATCAGAGCAGATCCG	
81684 of <i>L.striatellus</i>	T7-81684-F	T7 promoter sequence+ CACCAATGAGCTACCTGAAAGGTCT	634bp
	T7-81684-R	T7 promoter sequence+ AAGGCAGCCACAACCTGGTCC	
82635 of <i>L.striatellus</i>	T7-82635-F	T7 promoter sequence+ CAAGCCTGCCGAAACAGCTGTTAAA	702bp
	T7-82635-R	T7 promoter sequence+ TTAACAATCATTACCATAGCTCGG	
85613 of <i>L.striatellus</i>	T7-85613-R	T7 promoter sequence + CCGAAATCCTTTATGAGGAAGTTGT	523bp
	T7-85613-R	T7 promoter sequence + CTCAGATGTTAACAAACCAGCGCTC	
86923 of <i>L.striatellus</i>	T7-86923-R	T7 promoter sequence + TGGCCCTTCATGGCTTGGCTCATTT	518bp
	T7-86923-R	T7 promoter sequence + AACATAATGCCATTAAATCCCGGTT	

2.4 dsRNA synthesis and quantification

Nucleotide sequence of the candidate genes for dsRNA treatment were applied to the BLOCK-iTTM RNAi Designer (<https://rnaidesigner.thermofisher.com/rnaiexpress>) to predict the candidate siRNA sites, and dsRNA sequences were designed to include at least three putative siRNA sites. Single-strand cDNAs of the target genes were synthesized from total RNA of the *L. striatellus* using the QuantiTect Reverse Transcription Kit (QIAGEN, Germany) according to manufacturer's instructions, and the target genes were amplified with a set of primers including T7 promoter sequence (5'-TAATACGACTCACTATAG-3') at 5'-end (Table 9). Using the amplified product as template, dsRNAs for the target genes were produced by Genolution Pharmaceuticals (Korea). The molecular size of each synthesized dsRNA was confirmed on a 1.8% agarose gel.

2.5 Delivery of dsRNA via *O. sativa* leaves

L. striatellus were ingested dsRNA by rice-mediated feeding RNAi method using the fabricated feeding chamber according to previously described (An 2017). The process was described in Chapter 2. In this experiment three hundred microliters of each dsRNA solution was dispensed over the reservoir and three rice leaves were placed over the dsRNA solution. Fifteen 4th instar nymphs of *L. striatellus* were infested over three

dsRNA-permeated leaves. The dsRNA treated leaves infested with *L. striatellus* in the feeding chamber was placed in growth chamber at 28 °C, 80% relative humidity, and 16:8 (light:dark) photoperiod.

2.6 Assessment of gene knockdown

dsRNA-fed *L. striatellus* were collected at 48h post treatment, and their total RNA was extracted using Qiazol lysis reagent (Qiagen Ltd., Crawley, UK) according to the manufacturer's instructions. qPCR was performed in a 20 µl reaction volume using the EvaGreen qPCR Mastermix (Applied Biological Materials Inc, Canada) and CFX96™ Real-Time system (BIO-RAD, USA) according to the manufacturer's instructions. The progress of PCR amplification was as following: 95 °C for 10 min, (95 °C for 15 sec, 58 °C for 15 sec, and 72 °C for 30 sec) x 40 cycles. The ADP ribosylation factor (ARF) was used for the calculation of fold change as a reference gene. The relative transcription levels were calculated by using the $2^{-\Delta C_t}$ method (Pfaffl 2001). Primers used in this experiment were listed in Table 10.

Table 10. Primer sequences used for qPCR.

Gene name	Forward primer	Reverse Primer
82412	GGCCGAAAGAAGTCAAGAAG	CTTGCACGATGTTGTGATTG
79234	ATCTTCAGACCCGACGCTAT	TGATGCAAGGAAGTCTCTGG
64132	TGATGCTCACACATCGAGAA	AAGGCTACGACCAAAGGCTA
87012	CTTCCTTCACTCCCTGGAC	AAATTCCGTATTCGCTTGG
81684	CGTGATCAAGGAAACTGTGG	CGAGATGTGCTTGTCCACT
86923	GAGAACATGCGTCGAAGTTG	TGCAGACATAGCCTCCTCTG
66429	CGAATGAACGATGCTGAAAT	TTATTGTTGCTCCTGGCATC
72955	AAGCCGGCTGAGAAGAAGTA	GCTCCCAGCAAACACTTGTA
75761	CATCGGCCACATAGTCAGTT	AGTCTCACCAGGCAGTCCA
79041	AAACGGCTACATCACGACAG	TCCTGATCCGTGAGAATCAA
76485	ACCTCGCTGGAGAAGTCCTA	TTCCATACCCAGGAAGGAAG
80690	CAGCTTGCCAGTTTGAACC	GCAACAAACCTGGTTGTTC
82635	GAGGAAGAACTGAAGAAC	CAGGTCGTCGCGTTCCAC
84691	CTGGTGCTCGACGAGGCTG	CTTGGCAGACATCGACCAC
85613	CATCTACTTGGTCCACCTC	GAGCACAGGCGTCTGAAAC
83821	AGAGCTGAACGAGAGAAG	AGCTCTGAAGTTGACATG
86301	ACTTTAGTTGATACCTTGAAAG	GGAATGAATAGACTTCGCTG
78749	CAACAGTTTGTCCGACTGAG	AATGTCCGACATCGGTCATG
ARF	TTGGACAGTATCAAGACCCATC	GCAGCAATGTCATCAATAAGC
NS3	GCAGAGCTCTACATTGTGCCA	AACGTGCACTAAGAGGTGGTTT

3. Result

3.1 Establishment of RSV-viruliferous SBPH

Total RNAs from 20 4th instar nymphs that fed on RSV-infected rice leaves were used to conduct RT-PCR for confirmation of RSV acquisition to the *L. striatellus*. RT-PCR was conducted with a set of primers specific to *L. striatellus*, COX2-F and COX2-R, yielded expected product of 502 bp PCR fragment for RSV-infected rice leaves. While, RSV-NS3-F and RSV-NS3-R specific to RSV yielded expected product of 443 bp PCR fragment from *L. striatellus* fed on RSV-infected rice leaves (Fig. 14). The method of rapid acquisition of rice stripe virus from infected leaves *in vitro* in SBPH had been reported (Li 2014). To verify the RSV transmission efficient we designed 24 h, 72 h and 7 days post infection on RSV-infected rice leaves. The results demonstrated that the RSV normally transmitted into *L. striatellus* through feeding for RSV-infected rice leaves all on 24 h, 72 h and 7 days.

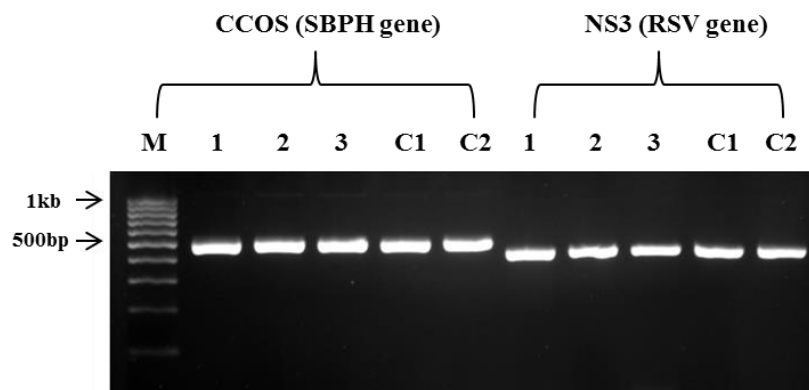


Fig. 14. RSV confirmation of *L. striatellus* by RT-PCR analysis. Lanes: M, 100 bp DNA ladder; 1: template with total RNAs from *L. striatellus* fed for 24 h on RSV-infected rice leaves; 2: template with total RNAs from *L. striatellus* fed for 72 h on RSV-infected rice leaves; 3: template with total RNAs from *L. striatellus* fed for 7 days on RSV-infected rice leaves; C1, C2: positive control.

3.2 Selection of the up-regulated genes in RSV-viruliferous SBPH for dsRNA synthesis

According to the previous RNA-seq analysis data in our lab (Lee 2013), the nucleotide sequence of 11 target SBPH genes which were up-regulated in RSV-viruliferous SBPH and dispersed in different GO groups were obtained (Table 11). Also seven SBPH genes related to RSV which have been reported up-regulated with the organ-specific response toward RSV were selected to proceed (Zhao 2016). And the transcriptional pattern in our RNA-seq data of these seven SBPH genes were listed in Table 12.

3.3 Validation of the sequencing results by qPCR

Total 18 selected genes from transcriptome analysis and previous publications were validated to the Illumina sequencing by qPCR in RSV-viruliferous SBPH. The transcription level of these 18 genes were calculated by qPCR with $2^{-\Delta Ct}$ method, and compared with those of Illumina sequencing result in FPKM value (Fig. 15). According to the validation result, we chose eight genes which were up-regulated their expression over 2 fold in RSV-viruliferous to synthesis dsRNA for RNAi experiment. The transcriptional pattern information of the eight genes were listed in Table 13 and simply renamed to

75761, 76485, 78749, 80690, 81684, 82635, 85613, and 86923. Among these eight genes, 75761, 76485, 81684 and 86923 were selected by transcriptome analysis between Non-viruliferous and RSV-viruliferous SBPH, and 78749, 80690, 82635 and 85613 were previously reported to be related with the organ-specific response toward RSV infection.

Table 11. The list of selected up-regulated genes in RSV-viruliferous SBPH

	Gene name	Target-ID	NOG_function	group	FPKM of NVLS	FPKM of RVL	R/N
1	82412	comp82412_c0_seq1	DEAD box ATP-dependent RNA helicase	A	30.37	138.50	4.56
2	79234	comp79234_c1_seq1	Tyrosine 3-monooxygenase	E	45.36	313.10	6.90
3	64132	comp64132_c0_seq1	GTP cyclohydrolase	H	33.41	152.10	4.55
4	87012	comp87012_c0_seq1	Ribosomal protein L23	J	73.41	315.20	4.29
5	81684	comp81684_c0_seq1	Cathepsin B protein	O	22.47	298.80	13.30
6	86923	comp86923_c0_seq1	Protein involved in cellular iron ion homeostasis	P	46.32	420.20	9.07
7	66429	comp66429_c0_seq1	Cytochrome P450 protein	Q	2.33	31.05	13.33
8	72955	comp72955_c0_seq1	Protein serine/threonine kinase	S	390.49	1852.00	4.74
9	75761	comp75761_c0_seq2	Structural constituent of cuticle protein	S	36.15	159.90	4.42
10	79041	comp79041_c0_seq1	Troponin C protein	T	30.66	197.00	6.43
11	76485	comp76485_c0_seq4	Actins	Z	97.24	442.70	4.55

NVL; Non-viruliferous SBPH, RVL; RSV-viruliferous SBPH, R/N; the FPKM ratio of

RVL to NVL. Group; function category of eggNOG database by using BLASTX.

Table 12. The transcription patten of selected genes from reported paper

	Gene name	Target-ID	NOG_function	group	FPKM of NVLS	FPKM of RVL	R/N
1	80690	comp80690_c0_seq3	Host cell factor	DK	0.53	0.55	1.05
2	82635	comp82635_c1_seq4	ND	K	2.74	9.90	3.61
3	84691	comp84691_c0_seq1	Atp-Dependent RNA helicase	A	1.50	3.65	2.43
4	85613	comp85613_c0_seq4	Cytochrome P450 protein	Q	1.43	4.76	3.33
5	83821	comp83821_c0_seq2	Vacuolar ATP synthase subunit g	C	11.63	11.61	1.00
6	86301	comp86301_c0_seq1	Serine protease inhibitor	V	2.51	3.23	1.29
7	78749	comp78749_c0_seq11	Peroxiredoxins, prx-1, prx-2, prx-3 protein	O	0.57	0.91	1.59

NVL; Non-viruliferous SBPH, RVL; RSV-viruliferous SBPH, R/N; the FPKM ratio of

RVL to NVL. Group; function category of eggNOG database by using BLASTX.

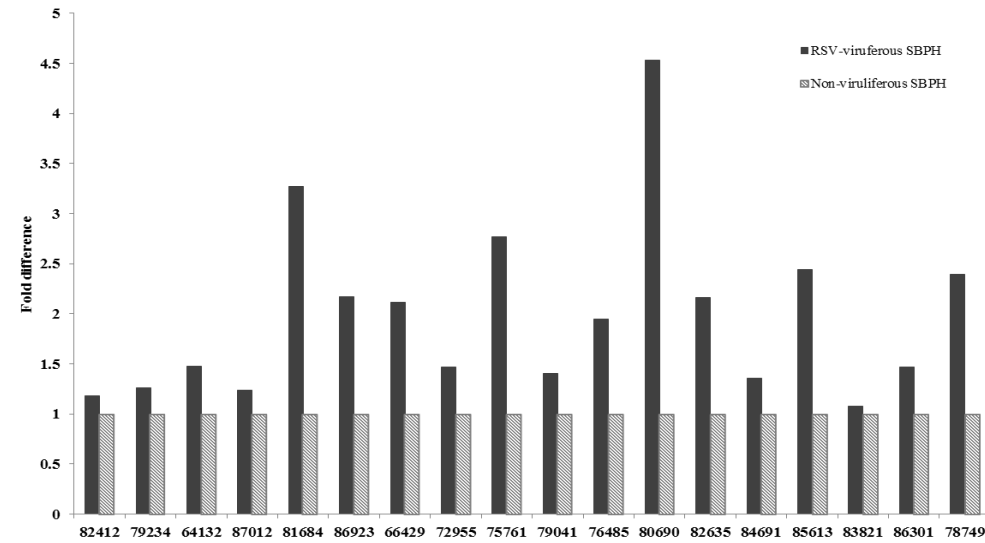


Fig. 15. Validation of the Illumina sequencing result by qPCR in RSV-viruliferous (RVL) and Non-viruliferous (NVL) SBPH. The transcription levels of 18 genes selected by NGS data and previous publications, were analyzed by qPCR to verify the Illumina sequencing result. The relative transcription levels were described in fold change.

Table 13. List of eight selected genes for dsRNA synthesis to suppress RSV replication in SBPH.

Contig rename	Contig number	Blast hit	Fpkm of NVL	Fpkm of RVL	R/N
75761	comp75761_c0_seq2	Structural constituent of cuticle protein	36.15	159.90	4.42
76485	comp76485_c0_seq4	Actins	97.24	442.70	4.55
78749	comp78749_c0_seq1	Peroxiredoxins, prx-1, prx-2, prx-3 protein	0.57	0.91	1.59
80690	comp80690_c0_seq3	Host cell factor	0.53	0.55	1.05
81684	comp81684_c0_seq1	Cathepsin B protein	22.47	298.80	13.30
82635	comp82635_c1_seq4	ND	2.74	9.90	3.61
85613	comp85613_c0_seq4	Cytochrome P450 protein	1.43	4.76	3.33
86923	comp86923_c0_seq1	Protein involved in cellular iron ion homeostasis	46.32	420.20	9.07

3.4 Detection of gene knockdown by feeding dsRNA

To investigate the efficacy of dsRNAs designed to knockdown the genes which are possibly responsible to the RSV replication in SBPH, qPCR was conducted to RSV-viruliferous SBPH which were ingested dsGFP and selected eight SBPH-derived dsRNAs (ds75761, ds76485, ds78749, ds80690, ds81684, ds82635, ds85613, and ds86923), respectively. Transcription levels of gene75761, 76485, 78749, 80690, 81684, 82635, 85613 and 86923 were all decreased after dsRNA treatment, suggesting that the target genes could silenced when ingested SBPH-derived dsRNAs by feeding RNAi system (Fig.16). The value of relative transcription level was accordant to the FPKM of each genes. And the relative transcription level of gene78749 was silenced to the greatest extent under ds78749 treatment.

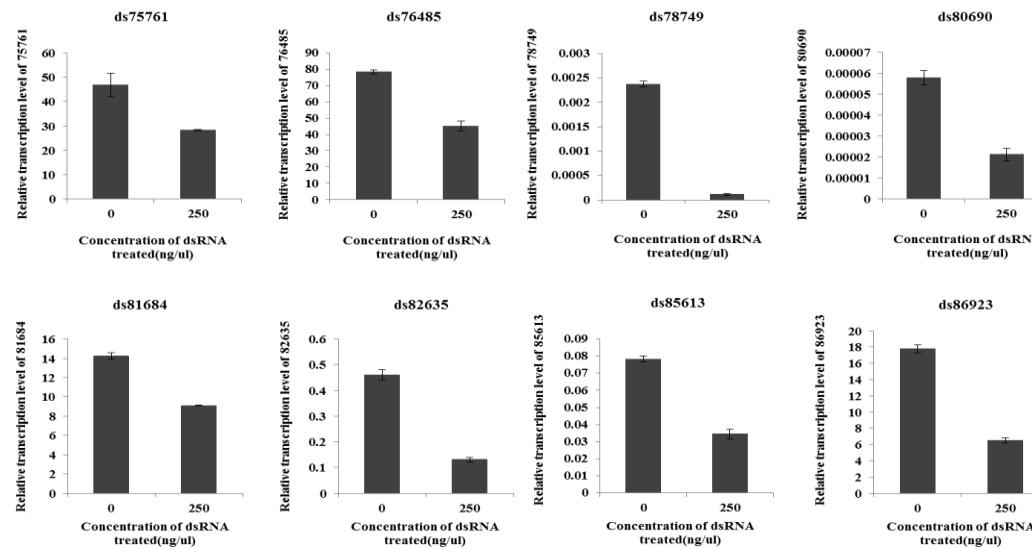


Fig. 16. Silencing of candidate genes of *L. striatellus* ingested SBPH-derived dsRNAs. Fifteen 4th instar nymphs of *L.striatellus* were fed on rice leaves steeped in dsGFP (Genolution Phamaceuticals,Korea) and eight SBPH-derived dsRNAs for 48 h. Relative transcription levels of eight SBPH-derived genes in RSV-viruliferous SBPH were measured by qPCR.

3.5 Reduced replication of RSV in *L. striatellus* ingested SBPH-derived dsRNAs

To investigate the suppression efficacy of RSV replication by dsRNA feeding method, qPCR was performed with template total RNA extracted from RSV-viruliferous SBPH ingested with dsRNA targeting 75761, 76485, 78749, 80690, 81684, 82635, 85613 and 86923, respectively. The transcription levels of RSV gene, NS3, was dramatically reduced by ds76485, ds78749, ds80690, ds81684, ds82635 and ds85613 treatment compared to those of untreated and dsGFP-treated *L. striatellus*. Especially, by ds78749, ds81684 and ds85613 treatment, relative transcription level of NS3 was reduced over 10 fold (Fig. 17).

Then ds78749, ds81684 and ds85613 which showed better efficiency than the others were selected to test at lower concentration of 50 ng/μl to investigate dose-dependency (Fig. 18). The result showed that not only in concentration of 250 ng/μl but also in lower concentration, transcription of NS3 was reduced significantly with ds78749, ds81684 and ds85613 treatment. The tendency of NS3 transcriptional suppression effect was proportionally related to the concentration of dsRNA.

For surveying the concentration range of dose-dependency with ds78749, ds81684 and ds85613 treatment, the lower concentration of 0.4, 2 and 10 ng/μl were ingested to RSV-viruliferous SBPH. The result showed that relative transcription levels of RSV NS3 gene were decreased in dose-dependent manner (Fig.19). These results suggested that

dsRNA feeding via plant could actually suppresses replication of RSV in *L. striatellus* through silencing with SBPH-derived dsRNA in RSV-viruliferous SBPH, especially ds78749, ds81684 and ds85613 these three dsRNA showed higher transcriptional seppression effect to RSV replication.

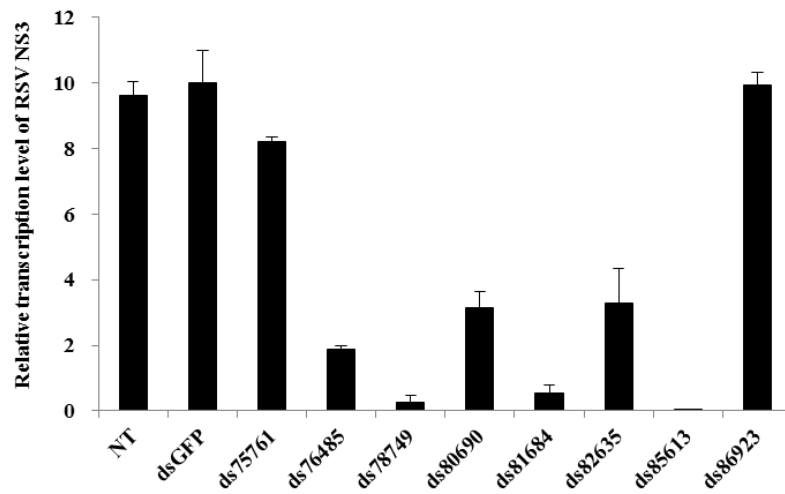


Fig. 17. Suppression of RSV replication in RSV-viruliferous SBPH ingested with SBPH-derived dsRNAs. Fifteen 4th instar nymphs of RSV-viruliferous *L. striatellus* were fed for 48h on rice seedling irrigated with 250 ng/μl dsRNA solution to suppress RSV replication. dsGFP was used for negative control. Relative titer of RSV in the RSV-viruliferous SBPH was measured by qPCR using primers for RSV NS3 gene. (NT= Negative control)

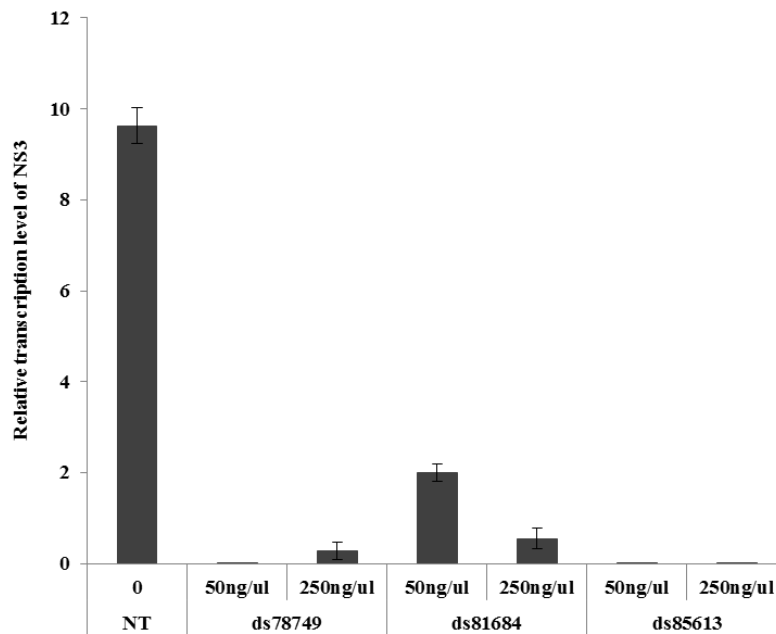


Fig. 18. Suppression of RSV replication in RSV-viruliferous SBPH ingested with selected SBPH-derived dsRNAs. Fifteen 4th instar nymphs of RSV-viruliferous *L. striatellus* were fed for 48 h on rice seedling irrigated with 50 and 250 ng/ μ l dsRNA solution to suppress RSV replication. dsGFP was used for negative control. Relative titer of RSV in the RSV-viruliferous SBPH was measured by qPCR using primers for RSV NS3 gene. (NT= Negative control)

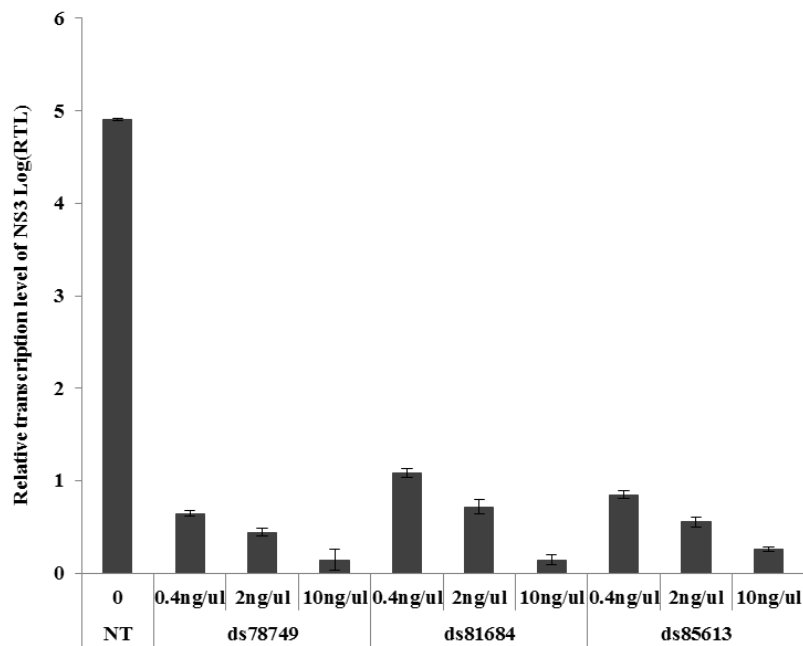


Fig. 19. Dose-dependent suppression of RSV replication in RSV-viruliferous SBPH ingested with selected SBPH-derived dsRNAs. Fifteen 4th instar nymphs of RSV-viruliferous *L. striatellus* were fed for 48 h on rice seedling irrigated with 0.4, 2 and 10 ng/μl dsRNA solution to suppress RSV replication. dsGFP was used for negative control. Relative titer of RSV in the RSV-viruliferous SBPH was measured by qPCR using primers for RSV NS3 gene. (NT= Negative control)

4. DISSCUSSION

For sucking insects, such as aphids, white flies and plant hoppers, dsRNA via artificial sap solution is a well-established system for feeding dsRNA (Shakesby 2009, Upadhyay 2011). In this experiment, we reconfirmed the RNAi feeding system for piercing-sucking small brown planthoppers based on dsRNA ingestion via rice leaves, and demonstrated that it is an efficient way to knockdown the target genes of *L. striatellus*. The *L. striatellus* ingested target dsRNAs along with host plant sap and tissues without any feeding avoidance, which frequently observed with artificial sap feeding. The results from this experiment revealed that three of the candidate genes were significantly knocked down and RSV replication could be suppressed by using the dsRNA feeding.

The gene 78749 indicated peroxiredoxins which are a ubiquitous family of antioxidant enzymes that also control cytokine-induced peroxide levels which mediate signal transduction in mammalian cells (Wood 2003). In insects, peroxiredoxins are believed to play an important role for protection against the toxicity of reactive oxygen species. Recently, it has been proposed that peroxiredoxins may play key roles in innate immunity and inflammation. Indeed, peroxiredoxins are evolutionarily conserved peroxidases able to reduce, with high rate constants, hydrogen peroxide, alkyl hydroperoxides and peroxynitrite which are generated during inflammation (Knoops 2016). According to

previous study it suggested that the ds78749 might indirectly affect the suppression of NS3 by inhibiting the protection to the toxicity of reactive oxygen species of *L.striatellus* nymphs although relationships between nuclear receptors and plant viruses in insect vectors have not been reported.

Cathepsin B protein coded by gene81684 was studied abundantly in insect and virus-transmitting vectors. The results showed that chemical inhibition of cysteine protease cathepsin B (cathB) restored the ability of aphids reared on turnip to transmit potato leaf roll virus in a dose-dependent manner, suggesting that the increased activity of cathB and other cysteine proteases at the cell membrane indirectly decreased virus transmission by aphids (Pinheiro 2017). But RNAi-mediated gene silencing of dengue virus (DENV) infection-responsive gene cathepsin B significantly reduced DENV replication in the salivary gland of *Aedes aegypti* (Sim 2012). A cathepsin B-like protease identified from *Helicoverpa armigera* reduced the survival time of baculovirus-infected *H. armigera* larvae, suggesting that this cathepsin B-like protease may possibly play a role in the recombinant virus in improving the rate of killing larvae. (Shao 2008). Like the *S. peregrina* cathepsin L-like protease, this cathepsin B played a normal role in the development of *H. armigera* (Zhao 2005, Yang 2006, Yang 2007), but had an insecticidal effect when indiscriminately expressed or expressed at levels far above those found under

normal physiological conditions. According to the previous studies, it is in accordance with our result which is cathepsin B protein not only has an insecticidal effect but also reduces the virus transmission to insect. This is the first report about cathepsin B protein affecting the RSV transmission to *L. striatellus*.

Gene85613 sequence was 94% identical to *Laodelphax striatellus* cytochrome P450 (CYP6CW1) (GeneBank Accession No.JX462960.1). This single P450 gene, CYP6CW1, was highly overexpressed (22.78-fold) in the resistance strain (YN-BPF) compared with the susceptible strain (YN) of *L. striatellus* has been reported (Zhang 2012). The P450s are encoded by a large supergene family with insect genomes sequenced to date containing from 46 to more than 150 P450 genes. Cytochrome P450 monooxygenases (P450s) can be involved in the detoxification and bioactivation of insecticides. Moreover, the transcriptional overexpression of P450 genes is often thought to enhance the metabolism of insecticides and appears to be a common phenomenon in the evolution of resistance development in insects. Monooxygenases of insects have several functional roles, including growth, development, feeding and protection against xenobiotics, including resistance to pesticides and tolerance to plant toxins (Scott and Wen 2001). In insects, P450 monooxygenase-mediated detoxification is one of the major mechanisms of insecticide resistance. P450s are among the major enzymes involved in conferring resistance to

different types of pesticides, and P450-mediated resistance typically results from the overexpression of one or more P450 genes. This resistance has been well documented in many insecticide-resistant insects, such as the house fly *M. domestica*, the brown planthopper *N. lugens*, the mosquito *C. quinquefasciatus*, the red flour beetle *Tribolium castaneum*, the fruit fly *D. melanogaster* and the greenhouse whitefly *Trialeurodes vaporariorum* (Carino 1992, Bogwitz 2005, Hardstone 2010, Zhu 2010, Bass 2011, Karatolos 2012). Also this cytochrome P450 has been researched abundantly about insecticide resistance, but the relationship with RSV was lacked. Our result suggests that the transmission of RSV could be suppressed by silencing of 85613 in *L. striatellus* and provides novel insight into the interactions between RSV and *L. striatellus*.

Insect vectors for plant pathogens are worldwide pests and pose a continuous threat to plants of economic importance. The damage caused by plant viruses is estimated in the billions of dollars each year globally. The majority of insect vectors are controlled using chemical insecticides that threaten the environment, human health and beneficial organisms. During the last decade, many research projects have employed RNAi and made it possible to unveil the function of new genes and developed a new research discipline in which the development of pesticide-free control methods against insect pests became realistic. Although there were many cases in which successful gene silencing in insect

vectors for plant pathogens were reported, very few cases investigated silencing genes that have direct role in virus transmission (Kanakala and Ghanim 2016). Our result just fills this gap and gives the three useful specific genes for the development of RNAi-based transgenic plants.

LITERATURE CITED

An, S. B., J. Y. Choi, S. H. Lee, Y. Fang, J. H. Kim, D. H. Park, M. G. Park, R. M. Woo, W. J. Kim and Y. H. Je (2017). "Silencing of rice stripe virus in *Laodelphax striatellus* using virus-derived double-stranded RNAs." Journal of Asia-Pacific Entomology **20**(2): 695-698.

Ansorge, W. J. (2009). "Next-generation DNA sequencing techniques." New biotechnology **25**(4): 195-203.

Araujo, R., A. Santos, F. Pinto, N. Gontijo, M. Lehane and M. Pereira (2006). "RNA interference of the salivary gland nitrophorin 2 in the triatomine bug *Rhodnius prolixus* (Hemiptera: Reduviidae) by dsRNA ingestion or injection." Insect biochemistry and molecular biology **36**(9): 683-693.

ASAI, T., M. FUKADA, S. MAEKAWA, K. i. IKEDA and H. KANNO (1983). "Studies on the Mode of Action of buprofezin I. Nymphicidal and Ovicidal Activities on the Brown Rice Planthopper, *Nilaparvata lugens* STAL (Homoptera: Delphacidae)." Applied entomology and zoology **18**(4): 550-552.

Bao, Y.-Y., Y. Wang, W.-J. Wu, D. Zhao, J. Xue, B.-Q. Zhang, Z.-C. Shen and C.-X. Zhang (2012). "De novo intestine-specific transcriptome of the brown planthopper

Nilaparvata lugens revealed potential functions in digestion, detoxification and immune response." Genomics **99**(4): 256-264.

Bass, B. L. (2000). "Double-stranded RNA as a template for gene silencing." Cell **101**(3): 235-238.

Bass, C., R. Carvalho, L. Oliphant, A. Puinean, L. Field, R. Nauen, M. Williamson, G. Moores and K. Gorman (2011). "Overexpression of a cytochrome P450 monooxygenase, CYP6ER1, is associated with resistance to imidacloprid in the brown planthopper, *Nilaparvata lugens*." Insect molecular biology **20**(6): 763-773.

Baum, J. A., T. Bogaert, W. Clinton, G. R. Heck, P. Feldmann, O. Ilagan, S. Johnson, G. Plaetinck, T. Munyikwa and M. Pleau (2007). "Control of coleopteran insect pests through RNA interference." Nature biotechnology **25**(11): 1322-1326.

Baumann, P. (2005). "Biology of bacteriocyte-associated endosymbionts of plant sap-sucking insects." Annu. Rev. Microbiol. **59**: 155-189.

Bautista, M. A. M., T. Miyata, K. Miura and T. Tanaka (2009). "RNA interference-mediated knockdown of a cytochrome P450, CYP6BG1, from the diamondback moth, *Plutella xylostella*, reduces larval resistance to permethrin." Insect biochemistry and molecular biology **39**(1): 38-46.

Bogwitz, M. R., H. Chung, L. Magoc, S. Rigby, W. Wong, M. O'Keefe, J. A. McKenzie, P. Batterham and P. J. Daborn (2005). "Cyp12a4 confers lufenuron resistance in a natural population of *Drosophila melanogaster*." Proceedings of the National Academy of Sciences of the United States of America **102**(36): 12807-12812.

Bolger, A. M., M. Lohse and B. Usadel (2014). "Trimmomatic: a flexible trimmer for Illumina sequence data." Bioinformatics **30**(15): 2114-2120.

Cahill, M., W. Jarvis, K. Gorman and I. Denholm (1996). "Resolution of baseline responses and documentation of resistance to buprofezin in *Bemisia tabaci* (Homoptera: Aleyrodidae)." Bulletin of Entomological Research **86**(2): 117-122.

Candy, D. and B. Kilby (1962). "Studies on chitin synthesis in the desert locust." Journal of Experimental Biology **39**(1): 129-140.

Carino, F., J. Koener, F. Plapp Jr and R. Feyereisen (1992). Expression of the cytochrome P450 gene CYP6A1 in the housefly, *Musca domestica*, ACS Publications.

Chen, J., D. Zhang, Q. Yao, J. Zhang, X. Dong, H. Tian and W. Zhang (2010). "Feeding - based RNA interference of a trehalose phosphate synthase gene in the brown planthopper, *Nilaparvata lugens*." Insect Molecular Biology **19**(6): 777-786.

Conesa, A., S. Götz, J. M. García-Gómez, J. Terol, M. Talón and M. Robles (2005). "Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research." Bioinformatics **21**(18): 3674-3676.

Culleres, D. B., J. Boesten, C. Bolognesi, A. Boobis, A. Buchert, E. Capri, D. Coggon, A. Hardy, A. Hart and H. Köpp (2007). "Opinion on Genotoxic and Carcinogenic Potential of buprofezin in the Context of the Human Risk Assessment. Scientific Opinion of the Panel on Plant Protection Products and their Residues (PPR)(Question No EFSA-Q-2007-130)." EFSA J **620**: 1-28.

De Cock, A. and D. Degheele (1998). buprofezin: a novel chitin synthesis inhibitor affecting specifically planthoppers, whiteflies and scale insects. Insecticides with Novel Modes of Action, Springer: 74-91.

Denholm, I., G. Devine and M. Williamson (2002). "Insecticide resistance on the move." Science **297**(5590): 2222-2223.

Denholm, I. and M. Rowland (1992). "Tactics for managing pesticide resistance in arthropods: theory and practice." Annual review of entomology **37**(1): 91-112.

Ding, S.-W. (2010). "RNA-based antiviral immunity." Nature Reviews Immunology **10**(9): 632-644.

Dong, Y. and M. Friedrich (2005). "Nymphal RNAi: systemic RNAi mediated gene knockdown in juvenile grasshopper." BMC biotechnology **5**(1): 25.

Falk, B. W. and J. H. Tsai (1998). "Biology and molecular biology of viruses in the genus Tenuivirus." Annual review of phytopathology **36**(1): 139-163.

Fang, Y., W. J. Kim, S.-H. Lee, J. H. Kim, K. B. Ha, D. H. Park, R. M. Woo, M. G. Park, J. Y. Choi and Y. H. Je (2017). "The transcriptomic responses of small brown planthopper, *Laodelphax striatellus* upon juvenile hormone agonist and antagonist treatment." Journal of Asia-Pacific Entomology **20**(2): 529-534.

Fire, A., S. Xu, M. K. Montgomery and S. A. Kostas (1998). "Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*." nature **391**(6669): 806.

Fire, A. Z. (2007). "Gene silencing by double-stranded RNA (Nobel Lecture)." Angewandte Chemie International Edition **46**(37): 6966-6984.

Gao, B., J. Wu, S. Huang, L. Mu and Z. Han (2008). "Insecticide resistance in field populations of *Laodelphax striatellus* Fallén (Homoptera: Delphacidae) in China and its possible mechanisms." International journal of pest management **54**(1): 13-19.

Grabherr, M. G., B. J. Haas, M. Yassour, J. Z. Levin, D. A. Thompson, I. Amit, X. Adiconis, L. Fan, R. Raychowdhury and Q. Zeng (2011). "Full-length transcriptome assembly from RNA-Seq data without a reference genome." Nature biotechnology **29**(7): 644-652.

Haas, B. and A. Papanicolaou (2016). TransDecoder (find coding regions within transcripts).

Haas, B. J. and M. C. Zody (2010). "Advancing RNA-seq analysis." Nature biotechnology **28**(5): 421-423.

Hammond, S. M. (2005). "Dicing and slicing." FEBS letters **579**(26): 5822-5829.

Hardstone, M., O. Komagata, S. Kasai, T. Tomita and J. Scott (2010). "Use of isogenic strains indicates CYP9M10 is linked to permethrin resistance in *Culex pipiens quinquefasciatus*." Insect molecular biology **19**(6): 717-726.

Hayano, Y., T. Kakutani, T. Hayashi and Y. Minobe (1990). "Coding strategy of rice stripe virus: major nonstructural protein is encoded in viral RNA segment 4 and coat protein in RNA complementary to segment 3." Virology **177**(1): 372-374.

Haynes, S., A. Darby, T. Daniell, G. Webster, F. Van Veen, H. Godfray, J. I. Prosser and A. Douglas (2003). "Diversity of bacteria associated with natural aphid populations." Applied and Environmental Microbiology **69**(12): 7216-7223.

Hibino, H. (1996). "Biology and epidemiology of rice viruses." Annual review of phytopathology **34**(1): 249-274.

Hibino, H., T. Usugi, T. Omura, T. Tsuchizaki, K. Shohara and M. Iwasaki (1985). "Rice grassy stunt virus: a planthopper-borne circular filament." Phytopathology **75**(8): 894-899.

Hogenhout, S. A., E.-D. Ammar, A. E. Whitfield and M. G. Redinbaugh (2008). "Insect vector interactions with persistently transmitted viruses." Annu. Rev. Phytopathol. **46**: 327-359.

Huang, H. J., J. Xue, J. C. Zhuo, R. L. Cheng, H. J. Xu and C. X. Zhang (2017). "Comparative analysis of the transcriptional responses to low and high temperatures in three rice planthopper species." Molecular Ecology **26**(10): 2726-2737.

Huvenne, H. and G. Smagghe (2010). "Mechanisms of dsRNA uptake in insects and potential of RNAi for pest control: a review." Journal of insect physiology **56**(3): 227-235.

Ishikawa, K., T. Omura and H. Hibino (1989). "Morphological characteristics of rice stripe virus." Journal of general virology **70**(12): 3465-3468.

Jespersen, S., P. Højrup, S. O. Andersen and P. Roepstorff (1994). "The primary structure of an endocuticular protein from two locust species, *Locusta migratoria* and *Schistocerca gregaria*, determined by a combination of mass spectrometry and automatic Edman degradation." Comparative Biochemistry and Physiology Part B: Comparative Biochemistry **109**(1): 125-138.

Ji, X., T. Ku, N. Zhu, X. Ning, W. Wei, G. Li and N. Sang (2016). "Potential hepatic toxicity of buprofezin at sublethal concentrations: ROS-mediated conversion of energy metabolism." Journal of hazardous materials **320**: 176-186.

Jing, P., L. Huang, S. Bai and F. Liu (2015). "Effects of rice resistance on the feeding behavior and subsequent virus transmission efficiency of *Laodelphax striatellus*." Arthropod-Plant Interactions **9**(1): 97-105.

Kahvejian, A., J. Quackenbush and J. F. Thompson (2008). "What would you do if you could sequence everything?" Nature biotechnology **26**(10): 1125-1133.

Kahvejian, A., J. Quackenbush and J. F. Thompson (2008). "What would you do if you could sequence everything?" Nature biotechnology **26**(10): 1125.

Kanakala, S. and M. Ghanim (2016). "RNA Interference in Insect Vectors for Plant Viruses." Viruses **8**(12): 329.

Karatolos, N., M. S. Williamson, I. Denholm, K. Gorman and C. Bass (2012). "Over-expression of a cytochrome P450 is associated with resistance to pyriproxyfen in the greenhouse whitefly *Trialeurodes vaporariorum*." PLoS One **7**(2): e31077.

Kim, J.-S., G.-S. Lee, C.-S. Kim, H.-S. Choi, S.-H. Lee, M.-K. Kim, H.-R. Kwag, M. Nam, J.-S. Kim and T.-H. Noh (2011). "Severe outbreak of Rice stripe virus and its occurring factors." The Korean Journal of Pesticide Science **15**(4): 545-572.

Kim, K.-H., E. Kabir and S. A. Jahan (2017). "Exposure to pesticides and the associated human health effects." Science of The Total Environment **575**: 525-535.

Knoops, B., V. Argyropoulou, S. Becker, L. Fert é and O. Kuznetsova (2016). "Multiple roles of peroxiredoxins in inflammation." Molecules and cells **39**(1): 60.

Langmead, B. and S. L. Salzberg (2012). "Fast gapped-read alignment with Bowtie 2." Nature methods **9**(4): 357-359.

Lee, J. H., J. Y. Choi, X. Y. Tao, J. S. Kim, W. Kim and Y. H. Je (2013). "Transcriptome analysis of the small brown planthopper, *Laodelphax striatellus* carrying rice stripe virus." The plant pathology journal **29**(3): 330.

Lenormand, T., D. Bourguet, T. Guillemaud and M. Raymond (1999). "Tracking the evolution of insecticide resistance in the mosquito *Culex pipiens*." Nature **400**(6747): 861-864.

Li, J.-M., Y.-R. Zhou, Z.-T. Sun, X. Wang, L. Xie and J.-P. Chen (2015). "Identification and profiling of conserved and novel microRNAs in *Laodelphax striatellus* in response to rice black-streaked dwarf virus (RBSDV) infection." Genomics data **3**: 63-69.

Li, J., I. B. Andika, J. Shen, Y. Lv, Y. Ji, L. Sun and J. Chen (2013). "Characterization of rice black-streaked dwarf virus-and rice stripe virus-derived siRNAs in singly and doubly infected insect vector *Laodelphax striatellus*." PLoS One **8**(6): e66007.

Li, J., Q. Chen, Y. Lin, T. Jiang, G. Wu and H. Hua (2011). "RNA interference in *Nilaparvata lugens* (Homoptera: Delphacidae) based on dsRNA ingestion." Pest management science **67**(7): 852-859.

Li, W. (2015). Fast program for clustering and comparing large sets of protein or nucleotide sequences. Encyclopedia of Metagenomics, Springer: 173-177.

Liang, D., Z. Qu, X. Ma and R. Hull (2005). "Detection and localization of Rice stripe virus gene products *in vivo*." Virus Genes **31**(2): 211-221.

Liu, S., D. Vijayendran and B. C. Bonning (2011). "Next generation sequencing technologies for insect virus discovery." Viruses **3**(10): 1849-1869.

Liu, W., S. Gray, Y. Huo, L. Li, T. Wei and X. Wang (2015). "Proteomic analysis of interaction between a plant virus and its vector insect reveals new functions of hemipteran cuticular protein." Molecular & Cellular Proteomics **14**(8): 2229-2242.

Mao, Y.-B., W.-J. Cai, J.-W. Wang, G.-J. Hong, X.-Y. Tao, L.-J. Wang, Y.-P. Huang and X.-Y. Chen (2007). "Silencing a cotton bollworm P450 monooxygenase gene by

plant-mediated RNAi impairs larval tolerance of gossypol." Nature biotechnology **25**(11): 1307-1313.

Mardis, E. R. (2008). "Next-generation DNA sequencing methods." Annu. Rev. Genomics Hum. Genet. **9**: 387-402.

Martín, D., O. Maestro, J. Cruz, D. Mane-Padros and X. Belles (2006). "RNAi studies reveal a conserved role for RXR in molting in the cockroach *Blattella germanica*." Journal of Insect Physiology **52**(4): 410-416.

Mercer, T. R., M. E. Dinger and J. S. Mattick (2009). "Long non-coding RNAs: insights into functions." Nature Reviews Genetics **10**(3): 155-159.

Merkling, S. H. and R. P. van Rij (2013). "Beyond RNAi: antiviral defense strategies in *Drosophila* and mosquito." Journal of insect physiology **59**(2): 159-170.

Merzendorfer, H. (2006). "Insect chitin synthases: a review." Journal of Comparative Physiology B **176**(1): 1-15.

Mutti, N. S., Y. Park, J. C. Reese and G. R. Reeck (2006). "RNAi knockdown of a salivary transcript leading to lethality in the pea aphid, *Acyrtosiphon pisum*." Journal of insect science **6**(38): 1-7.

Nagata, T. (1986). "Timing of buprofezin application for control of the brown planthopper, *Nilaparvata lugens* Stal (Homoptera: Delphacidae)." Applied entomology and zoology **21**(3): 357-362.

Otuka, A., M. Matsumura, S. Sanada-Morimura, H. Takeuchi, T. Watanabe, R. Ohtsu and H. Inoue (2010). "The 2008 overseas mass migration of the small brown planthopper, *Laodelphax striatellus*, and subsequent outbreak of rice stripe disease in western Japan." Applied Entomology and Zoology **45**(2): 259-266.

Patel, R. K. and M. Jain (2012). "NGS QC Toolkit: a toolkit for quality control of next generation sequencing data." PloS one **7**(2): e30619.

Pener, M. P. and T. S. Dhadialla (2012). "An overview of insect growth disruptors; applied aspects." Adv. Insect Physiol **43**: 1-162.

Pfaffl, M. W. (2001). "A new mathematical model for relative quantification in real-time RT-PCR." Nucleic acids research **29**(9): e45-e45.

Pinheiro, P. V., M. Ghanim, M. Alexander, A. R. Rebelo, R. S. Santos, B. C. Orsburn, S. Gray and M. Cilia (2017). "Host plants indirectly influence plant virus transmission by altering gut cysteine protease activity of aphid vectors." Molecular & Cellular Proteomics **16**(4 suppl 1): S230-S243.

Pitino, M., A. D. Coleman, M. E. Maffei, C. J. Ridout and S. A. Hogenhout (2011). "Silencing of aphid genes by dsRNA feeding from plants." PloS one **6**(10): e25709.

Power, A. G. (2000). "Insect transmission of plant viruses: a constraint on virus variability." Current opinion in plant biology **3**(4): 336-340.

Ramirez, B.-C. and A.-L. Haenni (1994). "Molecular biology of tenuiviruses, a remarkable group of plant viruses." Journal of general virology **75**(3): 467-475.

Roberts, A. and L. Pachter (2013). "Streaming fragment assignment for real-time analysis of sequencing experiments." Nature methods **10**(1): 71-73.

Roush, R. T. and J. A. McKenzie (1987). "Ecological genetics of insecticide and acaricide resistance." Annual review of entomology **32**(1): 361-380.

Scott, J. G. and Z. Wen (2001). "Cytochromes P450 of insects: the tip of the iceberg." Pest management science **57**(10): 958-967.

Shakesby, A., I. Wallace, H. Isaacs, J. Pritchard, D. Roberts and A. Douglas (2009). "A water-specific aquaporin involved in aphid osmoregulation." Insect biochemistry and molecular biology **39**(1): 1-10.

Shao, H.-L., D.-J. Dong, J.-D. Hu, J.-X. Wang and X.-F. Zhao (2008). "Construction of the recombinant baculovirus AcMNPV with cathepsin B-like proteinase and its

insecticidal activity against *Helicoverpa armigera*." Pesticide biochemistry and physiology **91**(3): 141-146.

Sim, S., J. L. Ramirez and G. Dimopoulos (2012). "Dengue virus infection of the *Aedes aegypti* salivary gland and chemosensory apparatus induces genes that modulate infection and blood-feeding behavior." PLoS pathogens **8**(3): e1002631.

Slama, K. (1971). "Insect juvenile hormone analogues." Annual review of biochemistry **40**(1): 1079-1102.

Sun, J., T. Hiraoka, N. T. Dittmer, K.-H. Cho and A. S. Raikhel (2000). "Lipophorin as a yolk protein precursor in the mosquito, *Aedes aegypti*." Insect Biochemistry and Molecular Biology **30**(12): 1161-1171.

Sylvester, E. (1980). "Circulative and propagative virus transmission by aphids." Annual review of entomology **25**(1): 257-286.

Takahashi, M., S. Toriyama, C. Hamamatsu and A. Ishihama (1993). "Nucleotide sequence and possible ambisense coding strategy of rice stripe virus RNA segment 2." Journal of General Virology **74**(4): 769-773.

Tang, M., L. Lv, S. Jing, L. Zhu and G. He (2010). "Bacterial symbionts of the brown planthopper, *Nilaparvata lugens* (Homoptera: Delphacidae)." Applied and environmental microbiology **76**(6): 1740-1745.

Tomoyasu, Y. and R. E. Denell (2004). "Larval RNAi in *Tribolium* (Coleoptera) for analyzing adult development." Development genes and evolution **214**(11): 575-578.

Toriyama, S. (1986). "Rice stripe virus: prototype of a new group of viruses that replicate in plants and insects." Microbiological sciences **3**(11): 347-351.

Toriyama, S., M. Takahashi, Y. Sano, T. Shimizu and A. Ishihama (1994). "Nucleotide sequence of RNA 1, the largest genomic segment of rice stripe virus, the prototype of the tenuiviruses." Journal of General Virology **75**(12): 3569-3579.

Turner, C., M. Davy, R. MacDiarmid, K. Plummer, N. Birch and R. Newcomb (2006). "RNA interference in the light brown apple moth, *Epiphyas postvittana* (Walker) induced by double - stranded RNA feeding." Insect molecular biology **15**(3): 383-391.

Uchida, M., T. Asai and T. Sugimoto (1985). "Inhibition of cuticle deposition and chitin biosynthesis by a new insect growth regulator, buprofezin, in *Nilaparvata lugens* Stål." Agricultural and Biological Chemistry **49**(4): 1233-1234.

Upadhyay, S. K., K. Chandrashekar, N. Thakur, P. C. Verma, J. F. Borgio, P. K. Singh and R. Tuli (2011). "RNA interference for the control of whiteflies (*Bemisia tabaci*) by oral route." Journal of biosciences **36**(1): 153-161.

Velculescu, V. E., L. Zhang, B. Vogelstein and K. W. Kinzler (1995). "Serial analysis of gene expression." Science **270**(5235): 484.

Wan, P.-J., S. Jia, N. Li, J.-M. Fan and G.-Q. Li (2014). "RNA interference depletion of the Halloween gene disembodied implies its potential application for management of planthopper *Sogatella furcifera* and *Laodelphax striatellus*." PLoS One **9**(1): e86675.

Wang, L., J. Fang and B. Liu (2008). "Relative toxicity of insecticides to *Laodelphax striatellus* (Fallén) (Homoptera: Delphacidae) and the resistance of field populations from different areas of East China." Acta Entomologica Sinica **51**(9): 930-937.

Wang, Y., H.-W. Fan, H.-J. Huang, J. Xue, W.-J. Wu, Y.-Y. Bao, H.-J. Xu, Z.-R. Zhu, J.-A. Cheng and C.-X. Zhang (2012). "Chitin synthase 1 gene and its two alternative splicing variants from two sap-sucking insects, *Nilaparvata lugens* and *Laodelphax striatellus* (Hemiptera: Delphacidae)." Insect biochemistry and molecular biology **42**(9): 637-646.

Wang, Y., H. Zhang, H. Li and X. Miao (2011). "Second-generation sequencing supply an effective way to screen RNAi targets in large scale for potential application in pest insect control." PLoS one **6**(4): e18644.

Wang, Z., M. Gerstein and M. Snyder (2009). "RNA-Seq: a revolutionary tool for transcriptomics." Nature reviews genetics **10**(1): 57-63.

Weisburg, W. G., S. M. Barns, D. A. Pelletier and D. J. Lane (1991). "16S ribosomal DNA amplification for phylogenetic study." Journal of bacteriology **173**(2): 697-703.

Whyard, S., A. D. Singh and S. Wong (2009). "Ingested double-stranded RNAs can act as species-specific insecticides." Insect biochemistry and molecular biology **39**(11): 824-832.

Wood, Z. A., E. Schröder, J. R. Harris and L. B. Poole (2003). "Structure, mechanism and regulation of peroxiredoxins." Trends in biochemical sciences **28**(1): 32-40.

Wu, W., L. Zheng, H. Chen, D. Jia, F. Li and T. Wei (2014). "Nonstructural protein NS4 of Rice stripe virus plays a critical role in viral spread in the body of vector insects." PloS one **9**(2): e88636.

Xiong, R., J. Wu, Y. Zhou and X. Zhou (2008). "Identification of a movement protein of the tenuivirus rice stripe virus." Journal of virology **82**(24): 12304-12311.

Xu, Y., L. Huang, S. Fu, J. Wu and X. Zhou (2012). "Population diversity of rice stripe virus-derived siRNAs in three different hosts and RNAi-based antiviral immunity in *Laodelphax striatellus*." PLoS One **7**(9): e46238.

Xue, J., X.-Q. Zhang, H.-J. Xu, H.-W. Fan, H.-J. Huang, X.-F. Ma, C.-Y. Wang, J.-G. Chen, J.-A. Cheng and C.-X. Zhang (2013). "Molecular characterization of the flightin

gene in the wing-dimorphic planthopper, *Nilaparvata lugens*, and its evolution in Pancrustacea." Insect biochemistry and molecular biology **43**(5): 433-443.

Yamamura, K., M. Yokozawa, M. Nishimori, Y. Ueda and T. Yokosuka (2006). "How to analyze long-term insect population dynamics under climate change: 50-year data of three insect pests in paddy fields." Population Ecology **48**(1): 31-48.

Yang, X. M., L. J. Hou, D. J. Dong, H. L. Shao, J. X. Wang and X. F. Zhao (2006). "Cathepsin B - like proteinase is involved in the decomposition of the adult fat body of *Helicoverpa armigera*." Archives of insect biochemistry and physiology **62**(1): 1-10.

Yang, X. M., L. J. Hou, J. X. Wang and X. F. Zhao (2007). "Expression and function of cathepsin B - like proteinase in larval hemocytes of *Helicoverpa armigera* during metamorphosis." Archives of insect biochemistry and physiology **64**(4): 164-174.

Zha, W., X. Peng, R. Chen, B. Du, L. Zhu and G. He (2011). "Knockdown of midgut genes by dsRNA-transgenic plant-mediated RNA interference in the hemipteran insect *Nilaparvata lugens*." PloS one **6**(5): e20504.

Zhang, F., H. Guo, H. Zheng, T. Zhou, Y. Zhou, S. Wang, R. Fang, W. Qian and X. Chen (2010). "Massively parallel pyrosequencing-based transcriptome analyses of small brown planthopper (*Laodelphax striatellus*), a vector insect transmitting rice stripe virus (RSV)." BMC genomics **11**(1): 303.

Zhang, Y., H. Guo, Q. Yang, S. Li, L. Wang, G. Zhang and J. Fang (2012). "Overexpression of a P450 gene (CYP6CW1) in buprofezin-resistant *Laodelphax striatellus* (Fall 6n)." Pesticide biochemistry and physiology **104**(3): 277-282.

Zhang, Y., Y. Wang, L. Wang, J. Yao, H. Guo and J. Fang (2016). "Knockdown of NADPH-cytochrome P450 reductase results in reduced resistance to buprofezin in the small brown planthopper, *Laodelphax striatellus* (fall 6n)." Pesticide biochemistry and physiology **127**: 21-27.

Zhao, W., L. Lu, P. Yang, N. Cui, L. Kang and F. Cui (2016). "Organ-specific transcriptome response of the small brown planthopper toward rice stripe virus." Insect biochemistry and molecular biology **70**: 60-72.

Zhao, X. F., X. M. An, J. X. Wang, D. J. Dong, X. J. Du, S. Sueda and H. Kondo (2005). "Expression of the *Helicoverpa* cathepsin b-like proteinase during embryonic development." Archives of insect biochemistry and physiology **58**(1): 39-46.

Zhu, F., R. Parthasarathy, H. Bai, K. Woithe, M. Kaussmann, R. Nauen, D. A. Harrison and S. R. Palli (2010). "A brain-specific cytochrome P450 responsible for the majority of deltamethrin resistance in the QTC279 strain of *Tribolium castaneum*." Proceedings of the National Academy of Sciences **107**(19): 8557-8562.

Zhu, Y., T. Hayakawa and S. Toriyama (1992). "Complete nucleotide sequence of RNA 4 of rice stripe virus isolate T, and comparison with another isolate and with maize stripe virus." Journal of General Virology **73**(5): 1309-1312.

Zhu, Y., T. Hayakawa, S. Toriyama and M. Takahashi (1991). "Complete nucleotide sequence of RNA 3 of rice stripe virus: an ambisense coding strategy." Journal of General Virology **72**(4): 763-767.

LI Shuo, WANG Shi-juan, ZI Jin-yan, WANG Xi and ZHOU Yi-jun (2014). "Rapid acquisition of rice stripe virus from infected leaves *in vitro* in small brown planthopper." Jiangsu Journal of Agricultural Sciences (2): 449-451.

ZHAO XiaoMing, JIA Pan, GUO Xin, LIU WeiMin, Ma EnBo and ZHANG JianZhen (2017). "Expression and Functional Analysis of Endocuticle Structural Glycoprotein Gene *LmAbd-5* in *Locusta migratoria*." Scientia Agrigultura Sinica **50**(10): 1817-1826.

ABSTRACT IN KOREAN

전사체 분석을 통한 유용 유전자의 RNAi 를 활용한 애멸구 및 벼줄무늬잎마름바이러스의 방제

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방영

초록

애멸구 *Laodelphax striatellus* (Fallén) 는 Hemiptera 의 Delphacidae 에 속하는 곤충으로, 경제적으로 중요한 흡즙성 곤충이다. 동남아시아, 시베리아, 유럽 등에 광범위하게 분포하며, 쌀 · 옥수수 · 밀 · 귀리 · 보리를 포함한 여러 농작물을 공격한다고 알려져 있다. 애멸구 는 벼 줄무늬잎마름바이러스(Rice stripe virus, RSV)를 순환 전파 방식으로 매개하기 때문에, 벼의 심각한 해충 중 하나이다. 벼가 RSV 에 감염되어 병에 걸리면, 잎에서 황백색의 줄무늬와

같은 병징이 관찰되고, 결실을 맺지 못하고 고사하게 된다. 이렇게 동남아시아에서 큰 문제가 되는 해충인 애멸구를 방제하기 위해 수십 년 동안 화학 살충제가 사용되었다.

Buprofezin 은 곤충 외골격의 주성분인 키틴 합성을 저해하여 곤충의 치사를 유도하는 Chitin synthesis inhibitor(CSI)계열의 곤충 성장 조절제(IGR)계열의 살충제로서, 애멸구에 매우 효과적인 것으로 알려져 있다. 이는 유익 곤충에게는 무해하고, 표적 해충에 대해 선택적이고 효과적으로 작용하기 때문에 특히 homoptera 해충 방제에 널리 사용되었다. 하지만, 애멸구를 방제하기 위해 Buprofezin 을 수십년간 사용하였기 때문에 최근 저항성이 보고되었다.

따라서 본 연구에서 Buprofezin 에 대한 애멸구 전사체 분석을 실시하였다. 200ppm 의 Buprofezin 을 4 령 애멸구에 침지법으로 처리하여 RNA 를 추출하였고, 그 결과 Buprofezin 을 처리한 실험군과 Buprofezin 을 처리하지 않은 대조군 샘플에서 각각 27,301,742 개와 26,848,684 개의 101 염기쌍의 paired-end short read 의 염기서열을 얻었다. Trinity *de novo* assembler 와 TransDecoder ORF finder 이용하여 23,817 개의 contig 를 포함하는 cDNA 라이브러리를 만들었다. 이 cDNA 라이브러리를 통해, Bowtie2 및 express 컴퓨터 프로그램을 이용하여 differential gene expression 을 얻었으며, 분석 결과

5 배 이상 down-regulated 되는 유전자는 170 개, up-regulated 되는 유전자는 144 개로 나타내어졌다.

GO enrichment 분석결과, 발현량이 2-5 배와 5-10 배 증가한 GO group 중, 각각 4 개 와 5 개의 GO group 에서 의미있는 유전자 발현의 차이를 확인하였다. 전사체 분석을 통하여 도출된 결과는 애멸구의 레퍼런스 유전자 염기서열로서 이용될 수 있고, GO enrichment 결과는 Buprofezin 과 관련된 애멸구의 유전자에 대한 유용한 정보를 제공함으로서 Buprofezin 과 같은 IGR 살충제의 분자생물학적 작용 기작 분석에 유용하게 사용될 수 있을 것이라고 판단된다.

새로운 해충 방제 방법을 개발하기 위해, 애멸구에 Buprofezin 을 처리하였을 때 특이적으로 발현이 감소하는 6 개의 유전자에 대해, dsRNA 를 활용하여 애멸구에 치사를 유도하는 연구를 수행했다. 합성한 dsRNA 중 3 종의 dsRNA 는 애멸구에게 7 일간 처리했을 때, 60%의 살충 효과를 나타내는 것을 알 수 있었다. 이러한 결과는 RNAi 를 기반으로한 해충 방제 방법의 가능성을 보여주었다.

RNAi 기법이 해충을 효과적으로 방제할 수 있는 새로운 방법으로 각광받고 있으며, 이는 매개충과 이들이 매개하는 식물 바이러스를 효과적으로 제어하는 역할도 한다. 따라서 본 연구에서는 RNAi 기법을 이용하여

애멸구에서 RSV 의 매개를 방제하기 위해, 보독 및 비보독 애멸구의 전사체를 분석하여 보독 애멸구에서 보다 높게 발현된 9 개의 유전자를 선발했다. RNAi 실험은 동일하게 벼를 통한 indirect feeding 방법으로 진행하였고, qPCR 결과 8 개의 dsRNA 중, 3 개의 dsRNA 가 매우 효과적으로 RSV 의 증식을 억제하는 것으로 나타났고, 이는 RSV 제어를 위한 새로운 방법이 될 수 있음을 시사한다.

High-throughput NGS 방법은, 비모델 곤충인 애멸구의 특정 RNAi 유전자를 선발하는데 유용한 정보를 제공하여, 이는 애멸구 방제와 RSV 를 억제에 대한 RNAi 기법을 이용한 기술은 새로운 해충 방제 전략으로 발전될 수 있음을 시사한다.

검색어: Rice stripe virus, *Laodelphax striatellus*, Buprofezin, RNA-seq, RNA interference, double-stranded RNA

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